

OSTEOPOROSIS

CONTEMPORARY ENDOCRINOLOGY

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OSTEOPOROSIS

PATHOPHYSIOLOGY
AND CLINICAL MANAGEMENT

Edited by

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and

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PREFACE

With the proliferation of books on osteoporosis in the last few years, one may reasonably ask “Why another?” Our answer is that the publication of this edition attempts to use a format that is not available in any other text or monograph in this field. Chapters dealing with basic science questions and findings are juxtaposed with chapters covering similar issues from a clinical standpoint. It is our hope that the integration of basic and clinical sciences in this format will allow readers from many disciplines to fully place in context the progress, or lack thereof, in the clinical and basic science arenas. At the same time, we hope to give basic scientists a better view of the state of the art as seen by their clinical colleagues, and vice versa.

We have attempted to cover as many of the relevant topics of interest as possible while keeping the length of the presented material reasonable. Traditional issues such as calcium supplementation and exercise are juxtaposed with updates in molecular pharmacology and imaging. The contributors are all recognized for their seminal contributions to the field; their rigor, enthusiasm, and (occasionally) focused uncertainty are readily apparent. It is hoped that the synergy and contrasts in the basic and clinical findings presented here will be cogent to the reader.

The quality of a multiauthor work such as this requires not only the effort and diligence of authors and editors, but also the patience and support of the publisher. We wish to thank Paul Dalgert and Craig Adams at Humana for their generous and timely input and guidance.

Eric S. Orwoll, MD
Michael Bliziotes, MD

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Human and Animal Studies of the Genetics of Osteoporosis

Robert F. Klein, MD and Tatiana Foroud, PhD

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INTRODUCTION

STUDIES IN HUMANS

STUDIES IN ANIMALS

THE APPLICATION AND RESEARCH FINDINGS IN CLINICAL SITUATIONS

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INTRODUCTION

Osteoporosis is a disease characterized by an inadequate amount and/or faulty structure of bone, resulting in fractures from relatively minor trauma. Although, osteoporotic fractures are most commonly observed among the elderly, the pathogenesis of osteoporosis starts early in life and involves the interaction of multiple environmental and genetic factors (1,2). Considerable past research has centered on the influence of reproductive, nutritional and/or lifestyle factors on the development of osteoporosis. With the advent of new molecular genetic approaches, the focus of research has recently shifted towards genetic factors. Genetic epidemiological studies provide convincing descriptive data demonstrating population and ethnic differences. In addition, studies of familial aggregation, familial transmission patterns, and comparisons of twin concordance rates consistently identify a significant portion of the vulnerability to develop osteoporosis as being inherited. Almost certainly, the development of osteoporosis will be found to involve a complex interplay between both genetic and environmental factors.

STUDIES IN HUMANS

Heritability of Skeletal Phenotypes

FAMILY STUDIES

Numerous family studies have demonstrated significant familial correlation for bone mineral density. An early study in a series of mother-daughter pairs estimated the heritability of radial bone mineral content to be 72% (3). More recently, Sowers et al. (4) estimated that genes account for 67% of the variability in femoral neck, 58% of the variability in Ward's triangle and 45% of the variability in trochanter (in a group of premenopausal sisters). A two-generation study of parents and their male and female

offspring (5) reported heritability estimates of 45–60% for various bone sites. Consistent heritability estimates were obtained using both sibling and parent-offspring correlations. After adjusting for lifestyle variables, such as calcium intake, alcohol intake, smoking, etc., heritability estimates of 46–60% were obtained for various bone sites. Similar findings were observed by Jouanny and colleagues (6) who found a correlation between the BMD of children over 15 and their parents ($r = 0.27$; $p < 0.0001$). Offspring had a 4.3-fold higher risk of low BMD if one parent had low BMD and an 8.6-fold higher risk when both parents had low BMD.

Several studies have also documented lower bone mineral density among individuals with a positive family history of osteoporosis (7–11). A comparison of premenopausal daughters of women with and without osteoporosis compression fractures found that the daughters of women with osteoporosis had 7% (approx $\frac{3}{4}$ –1 standard deviation change) lower bone mineral content at the lumbar spine ($p = 0.03$) as compared to the daughters of women without postmenopausal osteoporosis (8). There were also suggestive differences at the femoral neck (5% reduction; $p = 0.07$) and femoral midshaft (3% reduction; $p = 0.15$). In a second study, Seemen et al. (9) compared the daughters of women with and without hip fractures and found that women whose mother had a hip fracture had lower bone density at the femoral neck ($p < 0.05$) and femoral shaft ($p < 0.001$) as compared to women without a positive family history. However, they were unable to detect reduction at the lumbar spine (9).

Danielson et al. (10) found higher heritability estimates for BMD at the hip, spine and calcaneus for premenopausal daughters ($0.50 < h^2 < 0.63$) as compared to postmenopausal daughters (all $h^2 < 0.53$). A recent study in a sample of Caucasian premenopausal women confirmed the negative correlation between the number of relatives with osteoporosis and BMD as well as the lower BMD association with the presence of any male first degree relatives with osteoporosis (11).

Lower BMD has also been found among elderly subjects with a positive family history of osteoporosis. A study in an elderly (60–89 yr) population of both white men and women found that individuals with a positive family history of osteoporosis had lower BMD than those with a negative family history (12). In particular, there was a stepwise decrease in BMD as the number of family members with a positive history of osteoporosis increased. Among men, a maternal history of osteoporosis was associated with lower hip BMD while positive paternal history was associated with lower spine BMD. In women, only a positive paternal history of osteoporosis was associated with specific reductions in BMD levels at the spine and hip. These findings were confirmed in a large study of white women 65 yr of age or older. In this sample, a maternal history of hip fracture doubled the risk of hip fracture, even after adjustment for bone density (13).

Due to the strong effect of age on bone mineral density, most studies have compared adult family members in order to establish the familiarity of bone density measures. However, in a study of children between the ages of 5 and 20, significant correlation between parent and child BMD were observed with father-child correlation of 83% and mother-child correlation of 58% (14). More recently, Ferrari et al. (15) estimated the heritability due to maternal descent only, which was estimated as one-half of the total heritability ($\frac{1}{2} h^2$). In this sample of prepubertal girls, all under the age of 12, a series of bone mineral content variables were measured. For lumbar spine, femoral neck and midfemoral diaphysis, estimates of heritability due to maternal descent ($\frac{1}{2} h^2$) ranged from 18–37%. These significant parent-offspring correlations suggest that heredity influences bone density even in children who have not yet attained peak bone density.

Several studies have ascertained multigenerational families to formally evaluate the evidence of particular genetic models of osteoporosis using the technique of segregation analysis. Ascertained through a proband with low bone density, seven families having a total of 37 members with osteopenia (corrected spinal Z scores < -2.0) were identified. Commingling analysis suggested spinal BMD Z-scores had a bimodal distribution, consistent with an autosomal dominant mode of transmission (16). Livshits et al. (17,18) examined two independent populations, the Chuvasha and the Turkmenians, from distinct regions of the former Soviet Union. In both samples families of various sizes were ascertained, without consideration of previous fracture history. Over 500 individuals were examined from each population and BMD measures from the average of the second and third phalange of the middle finger were used for all analyses. Formal segregation analyses performed in the two populations analyzed separately and then in a joint analysis were consistent with a single major gene affecting both compact and cancellous BMD, suggesting a locus with pleiotropic effects. More recently, Cardon et al. (19) reported the ascertainment of eight families identified through a proband under the age of 35 yr with a history of two or more fractures and spinal bone density at least 2.5 standard deviations below mean for age and gender. Formal segregation analysis performed for the spinal BMD phenotype in these families was consistent with a major gene having codominant inheritance.

TWIN STUDIES

Bone Density. Twin studies have consistently demonstrated a genetic contribution to bone density. An early twin study examining a series of black and white, juvenile twins of both sexes identified heritability of both radial bone mass and bone width with heritability estimates of 75 and 77%, respectively (20). A subsequent juvenile twin study (21) found a somewhat estimate of heritability (47%) for cortical bone (radius); however, the spine BMC heritability estimate was 88%.

Confirmation of the early twin studies by Smith et al. (20), was found by Moller and colleagues (22) in elderly male and female patients. In this elderly sample, heritability for metacarpal total width was 77% and 78% for cortical width. Further evidence of significant BMD heritability was obtained in sample of pre- and postmenopausal males and females (23), in which significant heritability estimates were obtained for lumbar spine (92%), femoral neck (73%), Ward's triangle (85%) and trochanter (57%) BMD, and suggestive evidence of heritability from forearm BMC (42%). In a sample of 171 female sibling pairs with a wide age range (25–80 yr), Slemenda et al. (24) found heritability for radius BMC (52%) and BMD (70%), L2-L4 BMD ($h^2 > 100\%$), femoral (88%), Ward's triangle (84%) and trochanter ($h^2 > 100\%$) BMD. The authors note that the heritability estimates may be inflated due to failure of the assumption of no gene interactions. They suggest that the consistent evidence across several studies (23,24) of extremely high heritability values may instead support gene interactions among a relatively small number of genes.

A large sample of 500 postmenopausal female twin pairs, aged 50–70 yr was used to confirm the heritability of numerous bone density measurements (25). Consistent heritability estimates were obtained over several sites including lumbar spine (78%), total hip (67%), femoral neck (84%), Ward's triangle (51%), distal forearm (61%), mid forearm (46%), and whole body (76%). Smith et al. (20) also examined a series of adult male twins and found substantially lower heritability for bone mass (49%) and bone width

(45%) as compared to the juvenile sample. Higher heritability estimates were obtained in a second sample of adult male twins (21) in which the cortical bone BMC heritability estimate was 75%.

Bone Loss and Fracture. The early study by Smith et al. (20) utilized only white adult males and found a genetic contribution to midshaft bone mass and width. A follow-up study of a subset of this cohort continued to find strong genetic effects on midshaft radial width; however, there was no evidence of a genetic effect on bone loss over the 16-yr interval between examinations, suggesting that environmental factors rather than genes have greater influence on the rate of bone loss with aging (26). A later study (27) in a large sample of older males (average age 63 yr) confirmed the more prominent role of environment rather than genetics in bone loss. In that study, a very similar intraclass correlation for bone loss in both monozygotic and dizygotic twins was observed, suggesting a significant role of common environment, especially smoking and alcohol consumption, on bone loss. Contradictory evidence was obtained in a study of male and female twins with a wide age range (28). In this sample, evidence of a genetic effect on rates of change in lumbar spine and ward's triangle was demonstrated although there did not appear to be a significant genetic effect on changes in femoral neck.

Recently, a large Finnish study was performed to compare the rate of nontraumatic fracture among both male and female monozygotic (MZ) and dizygotic (DZ) twins born before 1946 (29). While the rate of twin concordance was greater among MZ twins as compared with DZ twins, for both male and female twin pairs, the magnitude of difference between the 2 types of twin pairs was quite small. The authors concluded from these that osteoporotic fracture as a phenotypic outcome was not strongly influenced by genetic factors. When these same data were then further analyzed by MacGregor et al. (30) using a variance components approach, they found that genetic factors accounted for approximately one-third of the variance in the liability to fracture at any of the body sites measured (either hip or spine) for both men and women.

HERITABILITY OF BIOCHEMICAL MARKERS OF BONE REMODELING

Few studies have been performed to evaluate the role of genetics on markers of bone formation and bone resorption. An initial study evaluated a sample of 140 pre- and postmenopausal female twin pairs for markers of bone formation (serum osteocalcin) and bone resorption (fasting urinary calcium:creatinine ratio and hydroxyproline:creatinine ratio). In this study only the bone formation marker, osteocalcin, had a significant genetic contribution (80%) to trait variability (31). These results were further examined using assays to detect circulating products of both type I collagen synthesis and degradation (32). Significant correlation between serum osteocalcin and carboxyterminal propeptide of type I procollagen (PICP) were found and, in concordance with the previous results of Kelly et al. (31), genetic factors were estimated to account for 95% of the variance in serum PICP levels. PICP is cleaved extracellularly from the carboxy-terminus of procollagen and its circulating levels are correlated with the bone collagen synthesis rate and osteoblast activity. Pyridinoline cross-linked carboxyterminal telopeptide of type I collagen (ICTP), which is cleaved during the degradation of type I collagen, was not found to be correlated with serum osteocalcin levels; however, the genetic contribution to the serum levels was estimated to be 64%.

In another sample of only postmenopausal twin pairs, markers of bone formation and resorption, were examined to identify those with evidence of genetic effects (33). The

three markers of bone formation, osteocalcin, PICP and serum bone-specific alkaline phosphatase (BAP) all had higher correlation among monozygotic as compared to dizygotic twin pairs, although significant genetic effects were found only for PICP (99%) and BAP (64%). Among the four markers of bone resorption that were examined, free D-pyridinoline, total D-pyridinoline, urinary type I C-telopeptide breakdown products (CrossLaps™), and urinary type I collagen cross-linked N-telopeptide (NTX), monozygotic twin correlation was significantly higher than dizygotic twin correlation for free D-pyridinoline and NTX; however, significant heritability was found only for free D-pyridinoline (86%). Thus, the results of this second study do not confirm a significant genetic role in osteocalcin; however, the serum PICP remained significantly heritable in both studies. Other findings found in one or the other study include significant heritability of BAP, and free D-pyridinoline.

While twins have been examined most commonly to estimate the heritability of biochemical markers of bone turnover, Livshits et al. (34) examined a sample of nuclear families from the Chuvasa region of the former Soviet Union and found that about 50% of the variation in PICP is attributable to genetic factors and about 40% of the variation in osteocalcin is due to genetic effects. Thus, a significant genetic contribution to PICP has been consistently reported; however, the apparent inconsistency of findings from one study to the other among the other biochemical measures may be due to the inherent technical difficulties and variability related to biochemical marker assays.

SUMMARY OF HUMAN STUDIES OF HERITABILITY

In summary, numerous studies have documented the substantial genetic contribution to BMD and peak BMD. However, genetic effects on the rate of bone loss have not been convincingly demonstrated. For these reasons, many studies seeking to identify genes underlying osteoporosis have relied primarily on the study of BMD, and in many cases on peak BMD, which appear to be the most heritable correlated phenotype.

Identification of the Genetic Determinants of Osteoporosis

CANDIDATE GENES STUDIES

For phenotypes with well-established heritability and a substantial genetic contribution to phenotypic variability, studies to identify the genetic loci influencing the traits are likely to be eventually successful. There are numerous experimental approaches that can be employed to identify genetic loci contributing to the risk for osteoporosis. One of the most commonly employed experimental designs is that of candidate gene analysis, which seeks to test the association between a particular genetic variant (i.e., allele), and a specific trait. Many of these candidate gene studies use population-based association methods. As applied to the study of osteoporosis, two samples are collected: a group of osteoporotic patients and a control group of non-osteoporotic subjects. The allele frequencies at a polymorphism within or near the candidate gene are then compared in the two groups. Ideally, the two groups should be matched so that they differ only in their disease status. In theory, evidence of differences in allele frequencies within the two populations (association) may be the result of linkage disequilibrium with the candidate gene or possibly with another gene in close proximity; however, in practice, the candidate gene is thought likely to be causative for disease.

Unfortunately, it is well recognized that admixture, heterogeneity, or stratification of a population may result in significant association, even when there is no susceptibility

locus in the chromosomal region (35). Therefore, the results of population-based association tests are often suspect and difficult to interpret (36). Despite the known limitations of population-based association studies it is a commonly used experimental design.

Those genes with known functions that fit current models of trait pathophysiology are often the first candidates for examination. Although a straightforward enterprise to perform such molecular studies in the laboratory, osteoporosis researchers employing candidate gene analysis face a dilemma. Given the complexity of skeletal physiology, there are likely to be a very large number of candidate genes responsible for the acquisition and maintenance of bone mass. Analysis of each one of these candidates, in isolation of the others, is likely to be prohibitive and difficult to interpret statistically and biologically (37–39). Similar to most other genetic studies of complex disease, osteoporosis researchers have studied numerous candidate genes, the most notable of which are summarized below. Candidate gene analyses have been performed in various populations using different polymorphisms within the same gene and have yielded, perhaps not surprisingly, inconsistent results across various experiments.

CANDIDATE GENES

Vitamin D Receptor. Due to its important role in the regulation of calcium homeostasis, the vitamin D receptor was one of the first candidate genes evaluated for its role in peak bone density. Using twin pairs, Morrison et al. (53) reported both linkage and association between a 3' untranslated region (UTR) polymorphism in the vitamin D receptor (VDR) and spine BMD. Initial results suggested that the B allele, defined by an absence of a *BsmI* restriction site in the 3' UTR, was associated with lower bone density and that the VDR locus might contribute as much as 75–80% of the variability in peak bone density, which would account for most, if not all, of the genetic contribution to BMD. In a series of over 300 unrelated healthy women, the majority of whom were postmenopausal, an association between the vitamin D receptor and lumbar spine and femoral neck BMD was observed. Among women with BMD values 2 standard deviations below the mean, there was an over-representation of the BB genotype. Subsequently the linkage data, although not the population association results, have been retracted (54).

Prior to the recent partial retraction, numerous studies in a variety of age groups were undertaken to evaluate the role of this 3' UTR VDR polymorphism on bone density and risk for osteoporosis. Work using an independent sample of premenopausal twin pairs (55,56) did not find any evidence to support the association between VDR and BMD. Conflicting results have been reported from a number of studies (11,57–68), although even those which were positive did not observe the magnitude of genetic effect reported in the initial Morrison et al. (53) publication. In fact, three reports have found the reverse association to that initially reported (i.e., the “b” allele was associated with lower BMD). A meta-analysis (69) of data from 16 studies found no significant association between the vitamin D receptor locus and bone density when the twin data of Morrison et al. (53), which was subsequently retracted (54), were excluded from the analysis.

The VDR sequence as originally reported by Baker et al. (70) contains two ATG (methionine) sequences in the first 4 amino acids. C/T polymorphism exists in the first potential ATG. Individuals with the T polymorphism start translation at the first methionine while those with the C polymorphism start translation at the second site resulting in a VDR protein that is 3 amino acids shorter. In light of the possibility that

this polymorphism could lead to functional consequences (i.e., it is possible that one form of the VDR might be translated more easily or be more stable in vivo than the other form), Gross et al. (71) performed an association study between this polymorphism and BMD in a population of Mexican American women. They found that individuals homozygous for the longer VDR protein (termed genotype ff) had significantly lower spine BMD than individuals homozygous for the shorter VDR protein (termed genotype FF). Heterozygous individuals (genotyped Ff) had intermediate spine BMD. A similar association was not observed in the same sample using femoral neck or forearm BMD. Association of BMD with the C/T polymorphism was not detected in a sample of premenopausal French women (72). Recently, a large sample of 535 Caucasian and African American sister pairs, which employed the start site polymorphism, did not find any evidence of linkage or association between VDR and BMD (73).

Collagen Type I Alpha I Gene. Abnormalities in type I collagen have been shown to result in osteogenesis imperfecta and mutations in the COL1A1 and COL1A2 genes, which encode the type I collagen proteins have been well-documented in patients with osteogenesis imperfecta. As a result, more subtle polymorphisms in the COL1A1 and COL1A2 were hypothesized to perhaps account, in part, for the genetic effects on bone mineral density (74,75).

Some studies of the regulatory region of COL1A1 have supported an association with bone mass (76,77). In two samples of white women, one from Aberdeen and the other from London, the majority of whom were postmenopausal, a common polymorphism was identified that results in a G to T substitution at the first base of a consensus site for the transcription factor Sp1 in the first intron of COL1A1 (76). A subset of women from both samples having vertebral compression fracture was compared to age and sex matched control samples and those with fracture had a significant over-representation of the heterozygous and homozygous substitution genotypes. In both samples, spine BMD values were significantly higher in women homozygous for the wild type allele (genotype termed SS) as compared to women who were heterozygous (Ss genotype). However, in the Aberdeen sample only, there was significant evidence of a gene dosage effect, with women homozygous for the substitution (ss genotype) having still lower spine BMD measurement. Results for hip BMD were not conclusive although in the Aberdeen sample a similar trend was observed with an apparent association of higher BMD values with the SS genotype. Further studies indicate that the evidence of association is much weaker with other COL1A polymorphisms, suggesting that in the Aberdeen sample at least, the association is particular to the Sp1 polymorphism (78).

The association between COL1A1 and bone mineral density has been confirmed in another sample (77). A gene-dose effect was observed at both femoral neck and lumbar spine with SS individuals having the highest BMD values, those with the Ss genotype having intermediate BMD values and ss individuals with the lowest. Similar to the UK studies, incident nonvertebral fractures were also related in a dose-dependent fashion to COL1A1 genotype. Several other studies have reported similar findings (79,80), while others have found much weaker evidence for association (81,82).

Similar to many reports of association between candidate genes and BMD, there have been a number of studies demonstrating no effect of the COL1A1 Sp1 polymorphism on BMD (83,84) or the rate of bone loss (85–92). These results clearly demonstrate that there is not universal agreement among all studies and in fact the effect of the COL1A1 Sp1 polymorphism, if present, is likely to be substantially less than originally estimated.

Estrogen Receptor. Decreased estrogen levels are known to be associated with an increase in bone turnover and bone loss, while estrogen replacement therapy ameliorates loss of bone. Therefore, the estrogen receptor is a reasonable candidate gene for osteoporosis. Recent studies by Kobayashi et al. (93) in a sample of postmenopausal Japanese women found an association of a particular estrogen receptor haplotype with bone density. In particular, absence of a *PvuII* polymorphism (denoted P) and presence of an *XbaI* polymorphism (denoted x) on both chromosomes (denoted PPxx) was found to be associated with significantly lower lumbar spine and total body BMD as compared to all other observed genotypes at these two loci. Some studies appear to confirm an association of the estrogen receptor with BMD (94,95). Subsequent studies have found that the nature of the association is often not consistent with the particular estrogen receptor haplotype initially reported nor with absence or presence of the two initially reported polymorphisms and the results vary depending on whether the women received postmenopausal hormone replacement therapy (96–102). In a series of other studies, investigators have not replicated any association between the estrogen receptor and BMD (103–106) or have not found the association to be maintained in postmenopausal women (107).

Apolipoprotein E. Apolipoprotein E (APOE) has been determined to be a susceptibility gene influencing the risk of Alzheimer disease (AD) in the general population. APOE has 3 common alleles, APOE*2, APOE*3, and APOE*4. Individuals with at least one copy of the APOE*4 allele are at increased risk of developing AD. In addition, this allele has also been associated with a four-fold increased risk of fracture among hemodialysis patients, especially those under the age of 80 yr (108).

The APOE genotype of women over 65 yr of age participating in the Study of Osteoporotic Fracture (SOF) was determined and compared to data collected both cross-sectionally and longitudinally. Women with at least one copy of the APOE*4 allele had significantly lower body weight and greater weight loss during the course of the study ($p < 0.01$). They also had a greater maternal history of fracture after age 50 as compared to women who did not have any copies of the APOE*4 allele. Longitudinally, women with an APOE*4 allele had higher rates of bone loss at both the total hip and femoral neck when compared with women without the APOE*4 allele ($p < 0.02$). Evidence of a gene dosage effect was observed when the rate of hip and wrist fracture was studied. Women homozygous for the APOE*4 allele had the highest rate of hip and wrist fracture with women heterozygous for the APOE*4 allele having intermediate rates of fracture. Importantly, there was no difference in mental status among women with and without the APOE*4 allele, suggesting that the higher fracture rate was not due to cognitive differences.

Further studies performed in pre-, peri- and postmenopausal women were performed to further analyze the relationship between APOE and bone loss (109). There was no evidence of a relationship between bone loss and APOE genotype among premenopausal women. However, bone loss among peri- and postmenopausal women was greater when they had at least one copy of the APOE*4 allele. Further analyses determined that the APOE*4 allele only increased bone loss among the peri- and postmenopausal women who were not currently using hormone replacement therapy. A study in a sample of postmenopausal Japanese women is consistent with a modest effect of the APOE*4 allele with low bone mass (110). Subsequent studies have not confirmed the role of APOE among postmenopausal women, even when hormone replacement therapy was considered in the analysis (111).

Other Candidate Genes. While several candidate genes have received the bulk of the attention of the osteoporosis research community, several other candidate genes have also been associated with bone mineral density. For example, there has been a report of an association of an interleukin-6 gene polymorphism with BMD (112) that has been refuted by at least one study (113). A one base deletion in the intron sequence 8 bases prior to exon 5 (termed 713-8delC) in the transforming growth factor beta 1 gene has been associated with very low bone mass in osteoporotic women and with increased bone turnover in both osteoporotic and normal women (114).

GENOME-WIDE LINKAGE STUDIES

Few genes influencing complex traits have been identified by the study of candidate genes alone. Therefore, researchers in the area of osteoporosis have utilized other types of experimental designs to identify genes contributing to the risk for osteoporosis. To improve the likelihood that a gene influencing osteoporosis might be identified, many investigators often proceed to search the genome, testing polymorphic markers evenly spaced on all chromosomes. A strength of the genome-wide approach is that it allows investigators to find susceptibility genes that are not obvious candidates based on current models of trait pathology.

One of the most important considerations for any type of study designed to identify genes for a complex trait is specification of phenotype. Researchers have employed various types of phenotypic definition in an effort to successfully identify genes contributing to osteoporosis. Low bone mineral density (BMD), independent of other factors such as falls and aging, is the strongest known determinant of osteoporotic fracture risk (40–42). Therefore, it is not surprising that many researchers have focused their efforts on the identification of genes contributing to BMD as a means to perhaps identify genes that also influence the risk of osteoporosis. The distribution of BMD is consistent with that expected from a quantitative or polygenic trait. That is BMD *per se* shows continuous variation, and discrete phenotypes are not generally discernible by studying the frequency distributions for bone density. Continuous variation is the consequence of the additive effects of genes (alleles) at multiple genetic loci that influence bone mass. Polygenic inheritance for bone density is consistent with previous findings that bone mass is influenced by multiple biochemical, mechanical and physiological systems, each of which may have its own genetic inputs. The challenge is to characterize these multiple genetic inputs (35).

Identification of the genes contributing to polygenic traits can be extremely complex, even for a phenotype such as BMD with substantial heritability. Therefore, several types of genetic studies have been employed to dissect the genetic contribution to BMD. One technique has been the identification of families with extreme BMD phenotypes. The rationale for such studies is that genes with a substantial contribution to BMD are more likely to be segregating in families with extreme BMD measurements. This strategy has been employed to ascertain families with either very high or very low BMD. An advantage of this approach is that statistical tests of linkage can be employed which model the genetic contribution to BMD as a single gene effect. Such studies typically employ parametric linkage analyses, the most powerful study design for the identification of genetic loci contributing to disease or in this instance, extreme BMD phenotypes. Unfortunately, there are several limitations to this particular experimental design. First, and perhaps most importantly, the genes found to contribute to the extreme

BMD phenotypes observed in these unusual pedigrees may not contribute substantially to the normal variation in BMD phenotype observed in the general population. A second limitation of the ascertainment of extreme pedigrees is their rarity in the population, resulting in great expense to identify such families.

An alternative to the identification of pedigrees with extreme phenotype is the ascertainment of families with members having BMD within the normal phenotypic range. In such pedigrees, BMD is inherited in a complex, non-Mendelian fashion, with multiple genes and environmental factors contributing to the phenotype. As a result, a particular model for BMD phenotype inheritance may be impossible to specify. In this instance, nonparametric linkage analysis, which does not specify a model for phenotypic inheritance, is the most powerful method for gene identification. This approach involves the identification of quantitative trait loci (QTL), which are genes whose alleles influence a quantitative trait. The overall genetic control of a quantitative trait generally results from the collective influence of many genes, each of which may contribute only a small amount to the genotypic variance, making their identification difficult. This previously daunting task has been made feasible through the recent implementation of technologies to identify genetic variation (polymorphisms) at landmark spots along the genome (marker loci) and the development of statistical methods to detect and genetically map the chromosomal locations of QTLs (37,43–47).

QTL analyses typically involve gathering a large number of related subjects thought to be segregating for genes that influence a given trait, and then following the transmission of allelic variants of marker loci from one relative to another. All statistical tests of linkage employing nonparametric linkage methods are based on identity by descent (IBD) marker allele sharing. Alleles are IBD if siblings inherit the same marker allele from the same parent. If the marker being tested is in close physical proximity to a gene influencing the phenotype, then siblings with similar phenotypic values would be expected to share marker alleles IBD. Conversely, siblings with dissimilar phenotypes would be expected to share fewer marker alleles IBD near the gene influencing the phenotype. An advantage of quantitative linkage methods as employed here is that no arbitrary cutoff for “high” or “low” phenotypic values is necessary; therefore, all siblings pairs are included in the analysis. QTL mapping can be a powerful strategy for the study of inherited diseases in both humans and animals.

Most human QTL analyses have, thus far, failed to detect genes with small to moderate effects and rarely, if ever, are designed to simultaneously assess multiple gene and environmental effects (48–51). Moreover, analyses designed to refine the chromosomal position of QTL may require finding an even larger number of families than that required to detect the initial linkage result. This is likely to be extremely costly, and may also create other problems. Families with different environmental exposures and genetic and ethnic backgrounds may enter into the sample, creating heterogeneity, and thereby decreasing the power to detect and localize a given QTL effect (52). The DNA sequence variants that are responsible for the QTL are unlikely to be immediately recognizable. In contrast to many qualitative, Mendelian traits where a discrete phenotypic difference is often the consequence of an inactivating mutation, the allelic variation responsible for quantitative traits probably has a subtler basis.

SIBLING PAIR APPROACH

Few large studies have been undertaken to identify novel genes underlying the risk for osteoporosis. Koller et al. (121) reported linkage to chromosome 11q12-13, a region

which was examined for linkage to peak BMD following the report of linkage of three Mendelian BMD-related phenotypes, autosomal dominant high bone mass, autosomal recessive osteoporosis-pseudoglioma and autosomal recessive osteopetrosis, to this chromosomal location. In the sample of 835 premenopausal Caucasian and African-American sisters, nonparametric linkage analysis methods supported a gene underlying peak BMD, with a maximum LOD score of 3.5 with femoral neck BMD. When the two races were analyzed separately, the Caucasian sample (364 independent sibling pairs) obtained a maximum LOD score of 2.78, while the African-American sisters (97 independent sibling pairs) had a maximum LOD score of 1.52.

Linkage analysis using BMD at other skeletal sites provided additional evidence for linkage to chromosome 11q12-13. The maximum lod score for the combined samples with Ward's triangle BMD was 2.84 with similar evidence found independently in the African-American (LOD=1.40) and Caucasian (LOD=1.46) samples. Linkage analysis with trochanteric BMD showed a maximum lod score of 1.87 in the combined sample with greater evidence found in the African-American (LOD=1.48) than in the Caucasian (LOD=1.16) sisters. Evidence for linkage to the 11q12-13 region was also found with lumbar spine and total body BMD, although the LOD scores were less than 2.0.

Subsequently, a 10 cM autosomal genome screen was completed in an initial sample of 429 Caucasian sister pairs (122). Multipoint nonparametric linkage analysis identified six chromosomal regions with LOD scores above 1.85 in the genome screen sample; however, only the linkage findings on chromosomes 1, 5, 6, and 22 were at or near a marker locus and therefore were pursued further in an expanded sample of 595 sister pairs (464 Caucasian, 131 African-American). The 11q region had already been pursued by genotyping an expanded sample of Caucasian and African-American sister pairs (121).

The results on chromosome 1q were the most promising of the genome screen. The initial genome screen resulted in a lod score of 3.11, which increased to 3.86 when additional Caucasian and African-American sister pairs were included in the analysis. Interestingly, this is not the same region of chromosome 1 reported by Devoto et al. (119), in a genome screen employing pedigrees ascertained on the basis of an osteoporotic proband.

Evidence of linkage to chromosome 5p also increased with the inclusion of additional Caucasian and African-American sibling pairs, from a genome screen LOD score of 1.9 to 2.2 after all available individuals were included in the analysis. Linkage to chromosome 6p was not substantially increased following the inclusion of both additional Caucasian and African-American sibling pairs. However, when only Caucasian sister pairs were included in the analysis the maximum LOD score was 2.1. Finally, the initial evidence of linkage to chromosome 22 decreased substantially with the inclusion of additional sibling pairs, suggesting that the initial linkage result was likely a spurious result. These linkage findings provide substantial evidence that genetic loci influencing the highly heritable bone mineral density phenotypes can be detected. These genetic studies have been completed in less than half of the final sample and suggest that samples of this size can be used to identify chromosomal regions in which to pursue high density genotyping.

More recently, Niu et al. (123) performed a genome wide screen for linkage to BMD in 153 sibpairs who were originally identified as extreme sibling pairs for blood pressure values. Using proximal forearm BMD, they obtained a peak LOD score of 2.15 over a very large region (>50 cM) on chromosome 2. This large region of chromosome 2 appears to include the region previously identified by Spotila et al. (120) using families ascertained through an individual with low BMD.

FAMILY STUDIES

High Bone Mass Families. One approach to identify genes for bone density is through families segregating apparently Mendelian forms of abnormal bone density. One such family was recently reported in which extremely high bone density (the converse of osteoporosis) appears to segregate as an autosomal dominant disorder (115,116). Affected individuals in this family have spine BMD measurements greater than 3 standard deviations above the mean without evidence of osteopetrosis or other osteosclerotic bone disorders. Affected individuals do not have clinical sequelae and the proband was identified fortuitously when high bone mass was noted following X-rays related to a car accident. The family has been extended and now includes 22 individuals, 12 of whom have the high BMD phenotype. Using a genome screen approach, linkage to chromosome 11q12-13 was identified with a LOD score of 5.74.

Genotyping of additional markers has localized the gene to a 30-cM interval. A systematic search for mutations that segregated with the high BMD phenotype identified an amino acid change in low density lipoprotein receptor-related protein 5 (LRP5) (123a). Of note, loci for the disorders osteoporosis-pseudoglioma (117) and autosomal recessive osteoporosis (118) have also been linked to this same region and loss-of-function mutations have recently been identified in families with osteoporosis-pseudoglioma syndrome (123b). LRP5 is a single pass membrane receptor involved in the Wnt canonical signaling pathway. Wnts are secreted factors that play critical developmental roles in cell fate determination and morphogenesis. The fact that sequence alterations in LRP5 can produce a spectrum of bone phenotypes indicates that this protein, and the Wnt signaling pathway through which it acts, may be exciting targets for the development of new therapeutic strategies. However, it is presently unclear as to whether allelic variation in LRP5 is an important determination of the distribution of BMD values observed in normal populations (123c).

Low Bone Mass Families. A series of families with multiple members having osteopenia has been used to evaluate candidate genes for osteoporosis (16) as well as to identify novel genetic linkage regions (119). Ascertained through a proband with low bone density, seven families having a total of 37 members with osteopenia (corrected spinal Z scores < -2.0) have been identified. Commingling analysis suggested spinal BMD Z-scores had a bimodal distribution, consistent with an autosomal dominant mode of transmission. Parametric lod score linkage analysis was performed in these families to evaluate three candidate genes: COL1A1, COL1A2 and the vitamin D receptor. Using an autosomal dominant model for the inheritance of spine BMD, with the allele predisposing for low BMD having a frequency of 0.01, linkage to all three candidate genes was excluded, with lod scores below -2.0 (16). Subsequently, parametric and nonparametric linkage analysis was performed in these 7 families using data from a genome screen (119). The maximum parametric LOD score ($Z_{\max} = 2.08$; $\theta = 0.05$) was obtained on chromosome 11q22-23 with the marker CD3D. Nonparametric sibling pair linkage analysis using 74 independent sibling pairs derived from these same 7 families supported linkage to chromosome 1p36 with a maximum LOD score of 3.51 at *DIS450* (multipoint LOD score = 2.29), chromosome 2p23-24 with a maximum lod score of 2.07 at *D2S149* (multipoint LOD score = 2.25), and chromosome 4qter with a maximum lod score of 2.95 at *D4S1539*. Extension of the study to include an additional 67 sibling pairs, each having at least one member with BMD values more than

Table 1
QTLs Contributing to Normal Variation in BMD in Humans

<i>Quantitative trait locus</i>	<i>Reference</i>
1p36	Devoto et al. (119)
1q21–23	Koller et al. (122)
2p23–24	Devoto et al. (119); Niu et al. (123)
4qter	Devoto et al. (119)
5q33–35	Koller et al. (122)
6p11–12	Koller et al. (122)
11q12–13	Koller et al. (115); Johnson et al. (122)

2 standard deviations below the norm, has increased the multipoint LOD score on chromosomes 1 to 3.01 (120).

SUMMARY OF HUMAN STUDIES OF LINKAGE

Various experimental approaches have been undertaken to identify genes contributing to the risk of osteoporosis. Extensive study of several promising candidate genes have yielded inconsistent results, suggesting that if these loci contribute to BMD, it is likely that their effect is relatively small and may be limited to particular populations. Various research groups using a variety of phenotypes including extreme and non-extreme families have undertaken genome-wide studies. While each study has identified unique chromosomal regions, there does appear to be some overlapping regions between studies, suggesting that these complementary approaches may provide insight regarding the genes contributing to BMD and which may therefore influence the risk of osteoporosis (Table 1).

STUDIES IN ANIMALS

Although recent clinical reports show promise, unraveling the very complex genetic basis of skeletal development will be difficult because of the genetic and cultural heterogeneity of the patient populations. One approach to this problem is the use of appropriate animal models to pinpoint candidate genes for more focused human investigation. Workers investigating determinants of bone mass in humans have limited ability to intervene in the genetics, personal environment, or skeletal biology of their subjects. In a complex disorder such as osteoporosis, experimental approaches that can either manipulate or hold constant biological variables that determine BMD provide a crucial opportunity to systematically examine the pathophysiologic processes that contribute to osteoporosis vulnerability. Animal research can help to elucidate possible roles of genetic and environmental constituents in the regulation of bone mass that might be otherwise difficult to untangle.

While the genetic basis for some extreme phenotypes might be due to the deletion of a gene or to an inactivating mutation, it is much more likely that extremes of BMD are due to subtle changes in gene expression, perhaps developmental genes early in life or possibly arising from allelic differences in untranslated regions of the genome that contain sequences controlling gene expression. Consequently, it will be very hard to devise a way of proving that a candidate gene really does underlie the phenotype. There may be no discernible sequence or expression difference to identify the gene, and even if there is, its presence does not prove etiological significance. While association studies can go some way to implicating a particular genetic locus, they can never be proof of a causal relation. For this a functional assay is needed: a way to alter the genetic sequence

and see whether this modification results in a different phenotype. Such experiments are possible only in animals and may be the sole way to understand how genetic differences result in individual variation in bone mass (124).

An ideal model that can be used for all studies in bone research does not exist. Whether or not an animal model is useful depends largely on the specific objectives of the study and frequently involves tradeoffs between such factors as realism, reproducibility of results and feasibility. Birds, mice, rats, rabbits, dogs, sheep, pigs, and nonhuman primates have all been the subject of experimental osteoporosis research (125–127). Each of these animal systems has its own advantages and disadvantages in regard to the following parameters: the similarity of skeletal metabolism and experimental bone disease to human processes, the time needed for breeding and for skeletal development, the cost of acquiring and maintaining the animals, and the ability to take advantage of both classical genetic techniques and the more recently developed molecular genetic techniques to introduce or eliminate specific genes. The obvious requirement for a reasonably detailed knowledge of basic genomic structure currently limits the choice for genetic animal models of osteoporosis to mice, rats and nonhuman primates.

Mice and rats are by far the most commonly used animals in bone research. Both mice and rats reach peak bone mass early in their life span and then undergo bone loss with aging (128–132). Following ovariectomy, a reduction in bone mass and strength occurs, that can be prevented by estrogen replacement (133–138). The SAM/P6 (senescence accelerated mouse/prone) mouse has low peak bone mass and develops fractures in middle and old age (139–146). It is the *only* experimental animal model with documented fragility fractures of aging. Histomorphometric studies of primates and humans yield very similar values (147). The nonhuman primate has both growing and adult skeletal phases. Peak bone mass occurs around age 9 yr in cynomolgus macaques (148) and around 10–11 yr in rhesus monkeys (149,150). Nonhuman primates experience decreased bone mass after ovariectomy (151–153), but the response to estrogen replacement has not been well-characterized. Nonhuman primates experience bone loss with age (154,155) but older animals also develop osteoarthritis with spinal osteophyte formation (156–158) that can obscure the accurate radiographic assessment of spinal bone mass (159). The extreme requirements for housing and care of nonhuman primates limit their use to a relatively small number of facilities.

Of the three currently available options, the mouse is arguably the model of choice because: (1) mice are much cheaper to house and easier to handle; (2) mouse genetic resources are quite extensive; and (3) once candidate genes are identified, the ability to manipulate them in mice and to deduce unambiguously their role in disease is unparalleled (160,161). Moreover, gene targeting has reached new heights in mice, but is barely on the horizon in other animals. With gene targeting perhaps as the ultimate arbiter for establishing cause-and-effect relationships between a candidate gene and osteoporosis susceptibility, the mouse is apt to remain the primary experimental model system for the foreseeable future (161,162).

Current Animal Research

INBRED STRAINS

A strain of a species is inbred when virtually every genetic locus is homozygous. What this means is that all individuals within an inbred strain share a set of characteristics that

uniquely define them compared to other strains. Typically, inbred strains are derived from 20 or more consecutive generations that have been brother x sister mated; the strain can then be maintained with this same pattern of propagation. Individual animals within an inbred strain are as identical as monozygotic twins. There are several qualities of inbred strains that make them especially valuable for research. The first is their long-term relative genetic stability. This is important because it allows researchers to build on previous investigations. Genetic change can occur only as a result of mutation within an inbred strain. A second important quality of inbred animals is their homozygosity because inbred strains will breed true. Once the characteristics of a strain are known they can be reproduced repeatedly allowing for replicate experimentation as well as for studies by other investigators. The influence of genotype upon a particular characteristic can be investigated by placing mice from several inbred strains in a common environment. Observed differences must then be, within limits, the consequence of genetic factors. By reversing this strategy, and placing mice from a single inbred strain in a variety of environments, it is possible to estimate the importance of environmental influences upon a parameter of interest. Thus, inbred animals can be used to determine whether genetic variation in the expression of a characteristic exists and the environmental malleability of the characteristic. Experiments with inbred strains also have some limitations. While strain differences are easily demonstrated, it is often very difficult to attach much meaning to these differences, because the genes and gene products involved are usually unknown. Because comparisons of mice from two or more strains do not usually provide any information about the nature of the genetic differences, crosses between genotypes must be used to analyze patterns of genetic influence (Fig. 1). Additionally, when using an inbred strain to investigate any type of phenomenon, it is important to be aware that the observations may be relevant only to that strain. Because an inbred strain differs from all others, there will be characteristics unique to it. It is therefore, important to use more than one strain to confirm that any observation obtained pertains to the species and not just to the strain studied.

Inbred mice of different strains exhibit marked differences in parameters of skeletal integrity. Kaye and Kusy (163) examined bone tissue from five inbred mouse strains (A/J, BALB/CByJ, C57BL/6J, DBA/2J, and PL/J). Although body weight was similar in all five strains, tibial bone mass, composition and biomechanical strength varied considerably. Using peripheral quantitative computed tomography, Beamer et al. (164) surveyed female mice from 11 inbred strains (AKR/J, BALB/cByJ, C3H/HeJ, C57BL/6J, C57L/J, DBA/2J, NZB/B1NJ, SM/J, SJL/BmJ, SWR/BmJ, and 129/J). This postmortem study found that phenotypically normal inbred strains of mice possess remarkable differences in total femoral BMD that were detectable as early as two months of age. Since these genetically distinct strains of mice were raised in the same controlled environment the observed differences are, in all likelihood, the result of genetic variation. Subsequent endocrinologic studies of F₂ progeny from a cross between C3H/HeJ and C57BL/6J found that those mice with the highest BMD also had the highest serum insulin-like growth factor-I (IGF-I) levels, whereas the F₂ progeny with the lowest BMD had low IGF-I levels (165). Although more than 35% of the variance in BMD for the F₂ mice could be attributed to serum IGF-I levels, definitive evidence of a causal relationship between circulating IGF-I and BMD will require more extensive functional studies using the two progenitor strains. These preliminary investigations clearly indicate substantial genetic regulation of BMD in mice. Modern genetic methods, such as selective breeding

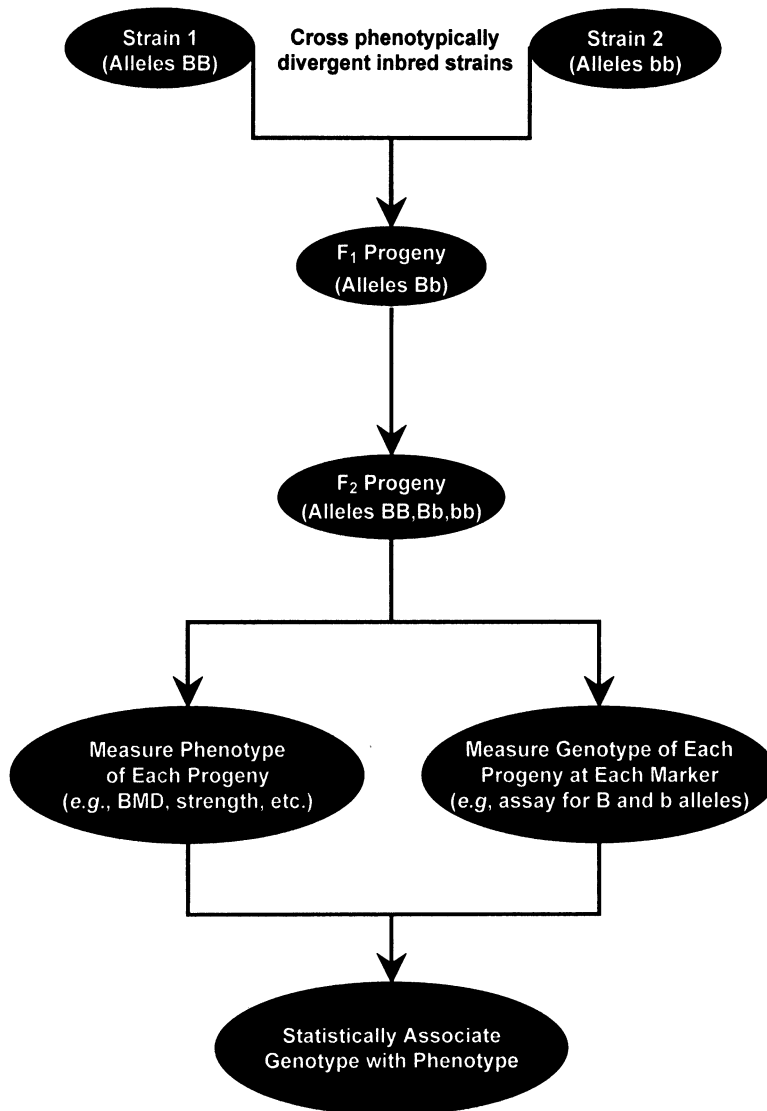


Fig. 1. Generation of murine recombinant inbred strains. Only four of the 19 autosome pairs from parental inbred strains “A” and “B”, and the assortment of chromosomes in the subsequent crosses derived from these strains, are shown. F_1 hybrids are genetically identical to each other, but individuals in the subsequent F_2 generation are not because of recombination events. RI strains also harbor recombinations but are homozygous at all loci as a result of the extensive inbreeding involved in their production.

and QTL analysis, can exploit these heritable strain differences to find and more directly evaluate the genetic linkage of osteoporosis-related traits (*vide infra*).

SINGLE GENE MUTATIONS

As described above, the study of inbred strains usually provides very little information about specific mechanisms of gene action. The analysis of single mutant vs normal genes is often a more effective approach. Comparisons between homozygous mutant mice and their “normal” homozygous wild-type and heterozygous litter mates may

provide considerable information on cellular mechanisms critical for discrete aspects of bone biology. Mouse enthusiasts have been breeding mice for centuries, thus maintaining spontaneous mutations. More than 140 spontaneous mutations affecting mouse bone morphology have been summarized by Green (166). For example, the short ear (*se/se*) mutation is associated with a number of skeletal defects, including reductions in long bone length and width, and the size of several vertebral processes, the absence of several small sesamoid bones and a pair of ribs, and impaired fracture healing (167–169). Recently, investigators discovered that the gene for bone morphogenetic protein-5 is disrupted in short-ear mice (170). There is also an expanding list of induced mutations in mice that cause recognizable skeletal pathology. Several lines of mice with mutations in type I collagen genes have been shown to develop a phenotype of skeletal fragility with extensive fractures of long bones and ribs (171–174). Mutations of a number of genes necessary for normal osteoclast development and/or function have been shown to result in osteopetrosis in mice (175–178).

QTL ANALYSIS

For a number of reasons, the laboratory mouse has proven to be an especially powerful tool for the identification and mapping of QTLs affecting complex polygenic traits (96). First, there is a wide range of phenotypic variation in genetically characterized animals (97), which is a prerequisite for QTL analysis. Second, factors such as short generation interval, ability to make designed matings and raise very large populations relatively inexpensively, and capacity to control or experimentally alter environmental factors enable QTL experiments in mice to have increased power, precision and flexibility. Third, the mouse has an extensively developed and well-organized molecular marker map, consisting of over 6500 easily typed PCR-based microsatellite markers (98) that exhibit allelic variation between lines. And fourth, the mouse is an anchor species in comparative genome maps representing homology among mammalian species (99). Once a chromosomal region harboring a murine QTL is identified, candidate chromosomal regions in humans where homologous QTLs may reside will be immediately apparent. Based on these attributes, research groups have successfully used mice in QTL detection studies for a number of quantitative traits, including obesity (100), body weight (94), and drug-seeking behavior (101).

Osteoporosis researchers are just now embarking on QTL analyses in large populations of mice in the hopes of obtaining a more complete picture of the polygenic control of bone mass and an improved understanding of the complex interactions and physiological mechanisms involved. Klein et al. (179) examined peak whole body BMD in female mice from a panel of 24 recombinant inbred (RI) strains of mice derived from a cross between C57BL/6J and DBA/2J progenitors (180; Fig. 2). The distribution of BMD values among the RI strains clearly indicated the presence of strong genetic influences, with an estimated narrow sense heritability of 35%. The pattern of differences in peak whole body BMD in the BXD strains were integrated with a large database of genetic markers previously defined in the RI BXD strains to generate chromosome map sites for trait locations. After correction for redundancy among the significant correlations, analysis of the RI strain series provisionally identified 10 chromosomal sites linked to peak bone mass development in the female. Using three additional independent mapping populations derived from the same progenitors, the chromosomal regions that harbor genes influencing peak BMD were confirmed on chromosomes 1, 2, 4, and 11.

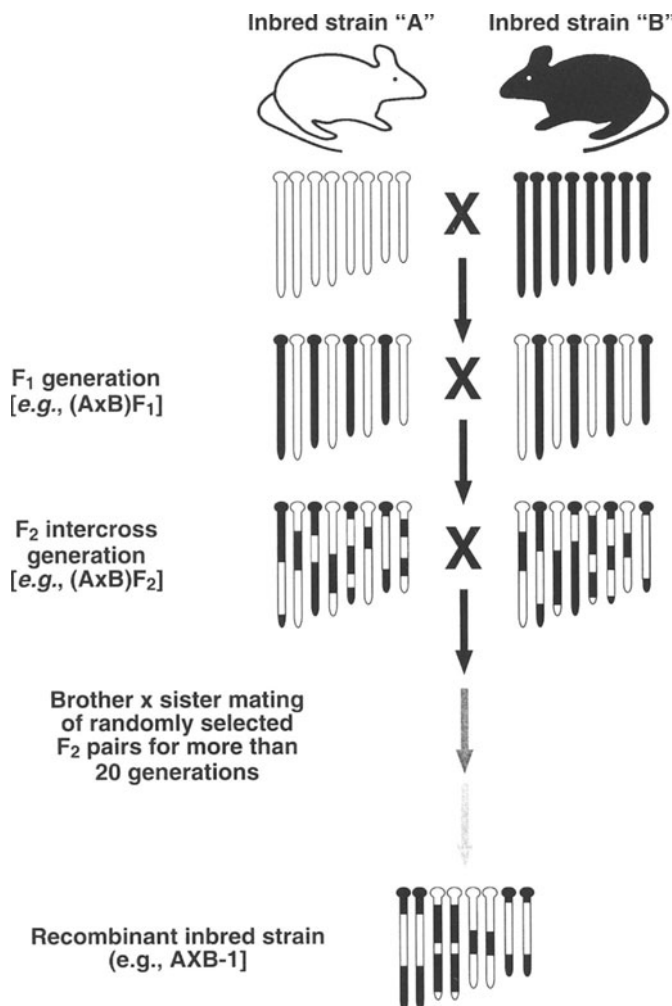


Fig. 2. Experimental steps in quantitative trait locus (QTL) mapping. Two different inbred strains are first crossed to produce genetically heterogeneous F₂ progeny. All of the progeny undergo phenotype assessment and then DNA samples are obtained to allow genotyping of each individual at multiple marker loci distributed throughout the genome. Statistical associations of markers and phenotypes are then performed to identify putative QTLs underlying the trait(s) of interest.

Several of the identified sites map near candidate genes of interest in skeletal biology. Beamer and colleagues examined femoral cortical bone density in 714 F₂ progeny from a C57BL/6J and CAST/EiJ cross (181). Significant QTL associations were found at 4 separate chromosomal sites. Two separate groups have performed F₂ intercrosses between SAMP6, an established murine model of senile osteoporosis, and other mouse strains of higher peak BMD and have mapped 5 QTLs significantly affecting spinal BMD (182) and two QTLs significantly affecting femoral cortical thickness (183). Results from these complementary studies should begin to define the landscape of the genetic regulation of BMD and help partition this quantitative trait into separate genetic components amenable for more detailed evaluation.

FUTURE DIRECTIONS

QTL analysis promises to identify the chromosomal position of many genes influencing osteoporosis-related traits. However, the ultimate goals of complex trait analysis—to identify coding sequences and to understand their biological roles at a molecular level—remain the major challenge. Initial QTL analyses on an adequately-sized F_2 intercross rarely succeed in narrowing map positions to less than 10–20 cM. This is because the phenotypes of individual animals are easily swayed by the influence of unlinked or environmental noise (161). Positional cloning of human disease genes has demonstrated that even when the position of a gene has been defined within one or two million base pairs and all the DNA sequences within that region have been isolated, identification of the relevant gene can still be a formidable task. Fortunately, new experimental strategies for fine QTL mapping, development of transgenic technologies, and more traditional approaches employing congenic strains, promise to eventually bridge the gap between cloning and disease.

QTL fine mapping involves careful analysis of recombinants within an interval previously found to contain the gene. For a compilation of the various experimental designs currently available, the reader is referred to an excellent recent review by Darvasi (184). One of the most attractive strategies exploits the high mapping resolution present in RI strains (recombinant inbred segregation test, RIST). Using the RIST design, a QTL of moderate effect, previously mapped to a 25 cM interval in an F_2 interstrain cross, can be mapped to a 1 cM interval with less than 1000 animals and only two stages of two generations each. Once the QTL has been resolved to such a narrow region, an examination of candidate genes within that region can take place and transform a conventional positional cloning strategy into a positional candidate approach.

Transgenic technology creates a very effective tool for analyzing the physiological roles of specific genes. A transgenic animal contains a segment of exogenous genetic material stably incorporated into its genome, resulting in a new trait that can be transmitted to further generations. Two widely used methods introduce exogenous genetic material into the genome: 1) microinjection of one-cell fertilized embryos, and 2) genetic manipulation of embryonic stem (ES) cells. In contrast to traditional “gain-of-function” mutations, typically created by microinjection of the gene of interest into the one-celled zygote, gene-targeting via homologous recombination in pluripotent ES cells allows one to precisely modify the gene of interest (185). Employing ES methodology, investigators have generated site-specific deletions (“knock-outs”), insertions (“knock-ins”), gene duplications, gene rearrangements, and point mutations. In addition to facilitating the study of known candidate genes, molecular complementation (transfer of specific genes) of selected phenotypes is a potentially important tool for gene identification. The recent success of transgenic technologies employing yeast artificial chromosomes (YAC transgenics) holds great promise for studying QTLs that influence a developmentally restricted phenotype which requires the transfer of both the locus and the long-range regulatory element(s) responsible for normal temporal or regional expression of the gene (186). Traditionally, the mouse has been considered the optimal animal model for conducting transgenic and gene-targeting experiments. Although investigators have succeeded in creating transgenic rats (187), the considerable time and expense involved limit the feasibility of widespread use of this animal model. Furthermore, gene-targeting technology to “knock-out” endogenous genes is currently feasible only in the mouse.

Classical transmission genetics can also be used to transfer a gene of interest from a donor strain or mutant onto the genetic background of an inbred strain. Using this approach, one is able to transfer regions containing risk or protective QTLs, or even multiple QTLs, onto appropriate background strains (Fig. 3). Such congenic strains are produced by repeated backcrossing to the background inbred strain and genotypic selection of the desired allele at a marker or markers at each backcross generation (188–190). After 7 backcross generations, the congenic and background strains can be expected to be about 98% genetically identical except for the transferred (introgressed) chromosomal region (190). The primary advantage of the congenics is that the influence of an individual QTL on any trait can be tested using the congenic vs background strain comparison at any level from the molecular to the physiological. Any differences found would strongly implicate a QTL in the introgressed chromosomal region as the cause of the differences. When there are several congenic strains for a given QTL, their differing sites of recombination can aid in attaining higher resolution mapping of the QTL with respect to neighboring markers. The near elimination of “genetic noise” due to unlinked loci greatly aids the search for candidate genes associated with each QTL, and for studies of differential gene expression (191). Ultimately, congenic strains can greatly facilitate positional cloning of a QTL. It can be anticipated that over the next one-two years, many genetic markers and almost all genes will be physically mapped in the human and mouse genomes. Consequently, knowing which genetic markers define a QTL region will automatically indicate which candidate genes are in the region. In addition, congenic strains provide an invaluable resource for further defining specific genes of interest and for in depth studies of the mechanisms by which they affect skeletal phenotype.

THE APPLICATION AND RESEARCH FINDINGS IN CLINICAL SITUATIONS

In light of the fact that specific genes that predispose to osteoporosis have yet to be identified, it is not currently possible to perform genetic testing for diagnosis or therapy of the disease. However, there are serious shortcomings in current strategies for diagnosis and therapy of osteoporosis. Identification of genes that predispose to osteoporosis will provide numerous opportunities to influence screening, diagnosis and therapy. How this will happen is, of course, dependent upon the number of genes that influence propensity to osteoporosis and their normal role in physiology. However, some benefits (and challenges) can already be anticipated from our current state of knowledge. Our discussion assumes that although it is likely that many genes influence predisposition to osteoporosis, there will be a limited number of genes that play important roles in osteoporosis.

Diagnosis

Currently diagnosis of osteoporosis is greatly dependent upon assessing BMD and other risk factors for fracture. Discovery of genes that play important roles in determining peak bone mass, rates of bone loss, and bone microarchitecture (i.e., trabecular thickness, number, etc.) may allow clinicians to divide the currently large population of osteoporosis patients on the basis of pathophysiology. This may have important implications for therapy since patients who have osteoporosis based on different pathophysiologies may respond differently to various therapies. Additionally, genetic studies may allow early identification of patients at high risk for osteoporosis when preventive

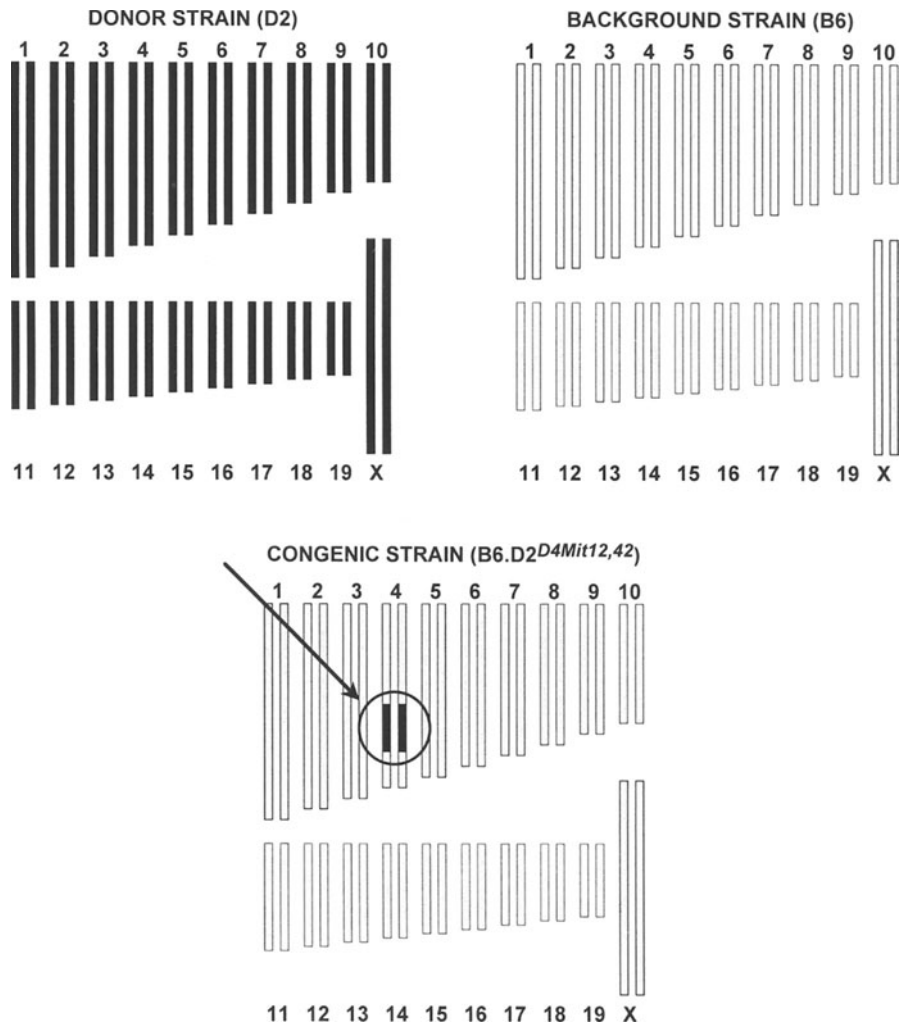


Fig 3. Congenic strain development involves the transfer of the region of a QTL from one progenitor strain (donor) to the other (background). In this example, a segment of chromosome 4 genomic material from D2 has been introgressed onto the B6 background strain through a process of repeated backcrossing and genotypic selection of the desired allele at each backcross generation. The production of congenic strains requires a great deal of time and effort, but the payoff is enormous. Because of the near elimination of “genetic noise” due to loci elsewhere in the genome, congenic strains can be invaluable tool for the identification of the precise gene(s) underlying a QTL.

measures, such as optimizing intake of calcium and vitamin D, may be more effective than later in the course of the disease.

The prospect of institution of early preventative strategies for this common disease raises the issue of genetic screening. In general, there are several conditions that must be met for a screening test to be useful (192). These conditions include the following: 1) the prevalence of the disease must be high in the screened population; 2) the test must be acceptable to the population screened; 3) there is an accepted therapeutic intervention

and early intervention improves outcome; 4) the cost of screening is acceptable; 5) the sensitivity and specificity of the test are sufficient to identify an acceptable percentage of affected individuals and a minimize the number of nondiseased individuals that are falsely identified; 6) the risk of the test is acceptable. Clearly, osteoporosis is a common disease and the prevalence, at least in postmenopausal Caucasian and Asian women, is high. Conditions 2–5 are dependent upon the type of genetic test employed. Although the physical risk of phlebotomy (or buccal smear) to obtain DNA is minimal, there is a risk of psychological harm in any genetic test. This issue has been extensively discussed elsewhere and will not be considered further, except to state that it needs to be considered and patients need to be properly informed before screening is initiated.

For any genetic screening test for osteoporosis to be useful it must not only meet the above criteria, but must also be a better screening test than measurement of BMD. While current guidelines (193) do not specifically use the term “screening,” BMD testing is recommended for all Caucasian women over the age of 65 and for all postmenopausal Caucasian women with additional risk factors regardless of age. Thus, densitometry is, in essence, already being used as a screening test. While the most cost-effective strategy has not been determined, BMD testing poses minimal risk, but a cost (approx \$30 for a peripheral measurement and \$130 for a central measurement). For any potential genetic test to be useful it must outperform BMD testing on either a cost or quality basis. Costs for genetic testing could be minimized if a limited number of functional polymorphisms could be tested in a automated fashion. However, it is unlikely that costs would be substantially below that of peripheral densitometry. Genetic tests might be more predictive of fracture than BMD if they provided information about risk factors other than BMD, such as trabecular microarchitecture, which is not available by other means. In any event, evidence that the genetic tests were better than BMD testing would have to be obtained empirically.

Perhaps the most important application of the discovery of genes that influence predisposition to osteoporosis is the potential effects on therapy. There are two potential mechanisms by which identification of these genes could have major effects on therapy: pharmacogenetics and identification of molecular targets/pathways. While much of the current emphasis in pharmacogenetics is focused on using genotypic information from an individual to determine if the patient will have an adverse affect from a particular drug, pharmacogenetics will eventually allow clinicians to use genetic information about a patient to choose the best therapy for that particular individual. As noted above, patients who have osteoporosis based on different pathophysiologies may respond differently to various therapies. Currently, clinicians choose a therapy, use it for 2 yr and reevaluate BMD to determine if the therapy increased BMD. If genetic testing allowed the clinician to determine which therapy would be most efficacious for a particular patient, therapy could be initiated with the most efficacious agent without delay or subjecting the patient to the side effects of a therapy that will not work for that patient.

In addition to pharmacogenetics, the discovery of genes that influence BMD and predisposition to osteoporosis will provide molecular targets for pharmacological intervention. This is particularly important in osteoporosis since all currently approved therapies target osteoclastic bone resorption. Ideally therapy should stimulate bone formation to replace lost bone, instead of simply preventing further loss. It is likely that some of the genes that influence predisposition to osteoporosis will have major effects on osteoblast regulation or function. Identification of these genes and the pathways that they

regulate will provide opportunities to develop agents that increase osteoblast function. Discovery of a new class of pharmacological agents that increased osteoblast activity and/or promoted de novo bone formation could lead to curative therapy for osteoporosis.

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The Genetics of Osteoporosis

Progress in Mice, not Man

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INTRODUCTION

Progress in the study of the genetics of osteoporosis is slow for several reasons. For example, the phenotype is poorly defined; fractures are too uncommon to allow credible detection of any association between genes that regulate a structural determinant of bone strength. Areal bone mineral density (aBMD) predicts bone strength, but it has not proven to be clinically useful in the identifying regulators of skeletal growth and aging, in part, because it is a two dimensional projection of a three dimensional structure. This measurement is too ambiguous to allow detection of the cell-, and surface-specific genetic determinants of bone size, geometry, and volumetric bone mineral density (vBMD). Even vBMD, the net result of changes in accrual and changes in bone size during growth and aging, and cortical thickness the net effect of periosteal and endocortical modeling and remodeling, may be too complex for detecting associations with specific genetically determined mechanisms. This complexity, in part, may explain why associations between aBMD and candidate gene polymorphisms are negative, contradictory or, at best, weak. Under 1–3% of the variance in aBMD is accounted for by any polymorphism, excluding their usefulness as a predictor of fracture or bone loss. Bone “loss” is not just resorptive “removal” of bone, it is the net result of the amount of bone resorbed on the endosteal (intracortical, endocortical, trabecular) surfaces and the amount formed on the periosteal surface. The net amount of bone resorbed is a function of the imbalance between the volume of bone resorbed and formed at each BMU and the rate of bone remodeling (activation frequency). Thus, examining the rate of bone loss using densitometry to identify genetic factors accounting for the variance in net bone loss

is fraught with problems. No trials have been done stratifying by genotype then randomization to placebo vs treatment within each genotype. Without this design, genotype specific differences in response to drugs, calcium supplementation, or exercise, may be due to covariates unevenly distributed in the genotype groups rather than the genotype.

Advances have occurred at a more reductionist level; in identification of gene regulation of osteoclastogenesis and osteoblast differentiation in animals, identification of loci on chromosomes associated with bone strength, identifying linkage in family studies between high bone mass and a region of a chromosome. Description of the age- and gender-specific means and variances of less ambiguous phenotypes such as bone length and periosteal growth (which determine bone size), and endocortical expansion or contraction (determining cortical thickness), trabecular numbers (determined at the growth plate) and thickness (determined by endosteal apposition) may be more fruitful provided that candidate genetic and environmental factors explaining part of this variance can then be identified.

WHY STUDY THE GENETICS OF OSTEOPOROSIS

This age-specific variance in regional bone mineral content (BMC) is large—1 standard deviation (SD) is about 10% of the mean. Thus, BMC at the 5th percentile is ~30% less than BMC at the 95th percentile. This variance is twice that seen in height where 1 SD in height is only 4% of the mean. Studies in twins and relatives of patients with fractures suggest that genetic factors account for 60–80% of this variance in bone mass (1,2). That is, the differences in bone mass between individuals of a given age is largely attributable to differences in their genes rather than differences in nonheritable environmental factors. Identification of pathogenetic mechanisms that account for *differences* in BMC between individuals is important because these pathogenetic mechanisms may be responsible for differences in bone fragility. Therefore, the purposes of identifying associations between candidate genes and bone fragility is to define causes of *variation* in the regulatory mechanisms responsible for *variation* in bone modeling and remodeling which determines *variation* in bone size, structure, geometry and so, *variation* in bone strength. This information may assist in identifying individuals at risk for fractures and individuals more sensitive to calcium supplementation, exercise, drugs, or corticosteroids, so that management can be focussed in a sensitive and specific manner.

Reduced BMC in patients with fractures relative to controls may be the result of reduced bone size or a reduced amount of bone within the periosteal envelope of the bone, i.e., reduced vBMD. These trait differences that may have their origins during growth or aging. Reduced periosteal apposition during growth or ageing may account for the smaller bone size in patients with fractures. Reduced vBMD may be due to thinner cortices produced by excessive growth-related endocortical expansion (itself due to excessive endocortical resorption or reduced endocortical formation) during prepubertal growth, reduced peripubertal endocortical apposition (in women), or due to an age-related increased intensity of endocortical remodeling (activation frequency), increased volume of bone removed or decreased volume of bone replaced in each BMU on the endocortical surface. Reduced trabecular numbers may have their origins pre or early post-natally, reduced trabecular thickness may have its origin pre- or peripubertally. During aging, remodeling increases on trabecular surfaces with age in women (and perhaps in men), and after menopause in women. Sex-specific changes occur in the

volume of bone removed and replaced within each BMU; greater resorption depth in women may result in loss of connectivity while a reduction in bone formation may favor trabecular thinning with less disruption to trabecular architecture in men.

Thus, the following questions arise; during growth, what is the age- and gender- and bone length-specific variance in periosteal apposition, endocortical resorption and formation during growth in males and females? What proportion of this variance is genetically determined and what specific genetic factors explain this genetic component of total variance? Are there specific genetic factors that identify persons with differences in periosteal apposition, and so, differences in bone size? Are there genetic factors influencing periosteal apposition and bone size that distinguish persons with and without fractures? Why is endocortical apposition sex- and site- specific? What genetic factors regulate and co-regulate the cellular events that establish the diameter of the bone, the proximity of these surfaces, and hence the thickness of the cortex, its distance from the neutral axis of the long bone, and so, bone strength? What is the variance in trabecular numbers at the growth plate, what is the size of the genetic component of variance, and, what genetic factors explain a proportion of the genetic variance. What genetic factors account for the age-specific increase in trabecular thickness during prepubertal and peripubertal growth? During aging, what genetic factors regulate and co-regulate continued periosteal apposition, endocortical and trabecular remodeling rate during adulthood, menopause and old age? What genetic factors influence the surface specific extent (activation frequency) of remodeling and the volumes of bone removed and replaced in the BMU, and thus BMU balance, the net amount of bone lost and its structural consequences during young adulthood, midlife and in old age in women and in men? Are there genetic factors identifiable in patients with fractures that explain any differences in the surface extent of bone remodeling and the nature of the remodeling imbalance that produces bone fragility?

Answers to most of these questions are not available. The purpose of this chapter is to describe problems that impede progress in the study of the genetics of osteoporosis, and to examine the role of genetic factors that influence the modeling and remodeling of the periosteal and endosteal (endocortical, trabecular, intracortical) surfaces of the skeleton. It is the absolute and relative movement of these surfaces that determine external bone size, the proximity of these surfaces to each other and hence the mass of bone, its geometry, macro- and micro- architecture, and so, its strength.

PROBLEMS IN DEFINING THE PHENOTYPE

The Uncommon Occurrence of Fracture

Detecting an association between the occurrence of fractures with a candidate gene influencing bone fragility is unlikely to be fruitful for several reasons. First, fractures are uncommon annual events, usually being ~1–3 per 100 women per year in the elderly and reaching ~10% per year in the highest risk groups. Therefore, detecting an association between the incidence of fractures and a candidate gene that explains part of the variance in bone strength is difficult because of the scarcity of fracture events. Likewise, detecting a greater concordance rate in fractures in identical versus nonidentical twins, or linkage of fractures with a genotype in family studies presents logistic problems because of the scarcity of fracture events.

Given that an association is detected, the multifactorial nature of fracture suggests that only a small proportion of fractures are the result of the structural abnormality produced by any one or more genotypes. Most fractures in the community are likely to be due to structural abnormalities produced by genetic and nongenetic factors quite unrelated to the single genotype being studied. That is, the attributable risk conferred by the gene is likely to be small. To be of value in predicting fractures *in the population*, the genotype must be common and have a large effect. If uncommon it will account for few fractures and will be difficult to detect.

Kannus et al. examined whether genetic factors explain variation in risk of fracture in a 25-yr follow up of 2308 monozygotic and 5241 dizygotic twin pairs; 786 subjects sustained an osteoporotic fracture (3). In women, the pairwise concordance rate for fracture was 9.5% (95% CI 5.3–15.5) in monozygotic, and 7.9% (5.2–11.4) in dizygotic pairs. In men, the figures were 9.9% (4.4–18.5) and 2.3% (0.6–5.7) leading the authors to conclude that susceptibility to osteoporotic fractures is not influenced by genetic factors.

Deng et al. reported the prevalence of Colles fractures (CF) is 11.8% in 2471 women aged 65 years, 4.4% in 3803 of their sisters and 14.6% of their mothers (17). The probability that a woman will suffer CF if her mother has, is 0.155, the probability that a sister will suffer CF given that her sister has, is 0.084. The relative risk (the ratio of the recurrence risk to prevalence), measures the degree of genetic determination, which is 1.3 for a woman with an affected mother and 1.9 for a woman with an affected sister. These parameters are consistent with a heritability of 0.25 for CF.

If a genotype is associated with increased fracture risk it should be more prevalent in fracture than non-fracture cases. Several candidate genes have been examined in this regard.

COLLAGEN TYPE I

Aerssens et al. found no evidence for a higher prevalence of VDR, COLIA1, and ER genotypes in 135 patients with hip fractures compared with 239 controls (4). Uitterlinden et al. reported that women with Ss or ss polymorphism of the COL1A1 are over represented in the 111 women with nonvertebral fractures (RR = 1.3) (5). For incident nonvertebral fractures the proportions of fracture cases in the SS, Ss, and ss groups were 5.4% (64/1194), 7.4% (39/526) and 13.8% (8/58); only the latter achieved significance (odds ratio, OR = 2.2). For prevalent vertebral fractures, the respective figures were 4.6% (44/949), 6.7% (28/420) and 4.5% (2/44) (NS). Even though the OR for nonvertebral fractures was statistically significant, only 8 of 111 women with nonvertebral fractures had the ss polymorphism, and 50 subjects with the ss polymorphism had no fractures.

The same investigators analyzed the combined influence of polymorphisms in the VDR and COLIA1 genes for fracture, 1004 postmenopausal women were studied (6). The “baT” VDR haplotype, constructed from three adjacent restriction fragment length polymorphisms, was over represented in fracture cases giving an OR of 1.8 (1.0–3.3) for heterozygous carriers and 2.6 (1.4–5.0) for homozygous carriers of the risk haplotype. The effect was similar for vertebral and nonvertebral fractures and was independent of BMD. Fracture risk was not VDR genotype-dependent in the COLIA1 “reference” group (genotype GG) while in the COLIA1 “risk” group (genotypes GT and TT) the risk of fracture was 2.1 (1.0–4.4) for heterozygous and 4.4 (2.0–9.4) for homozygous carriers of the VDR risk haplotype. The authors infer that VDR and the COLIA1 polymorphisms are genetic markers for osteoporotic fracture in women, independent of BMD and that

interlocus interaction is likely to be an important component of osteoporotic fracture risk. The attributable risk was not reported.

Weichetova et al. report that polymorphism of an Sp1 binding site in the first intron of the COL1A1 predicts wrist fracture risk (7). Among 126 postmenopausal women with low bone mass and one or more wrist fractures, and 126 postmenopausal women with low bone mass without fractures, femoral neck BMD was the strongest predictor of prevalent wrist fracture. COL1A1 genotyping strengthened prediction; Ss had 2 times the risk of SS, ss had 2.8 times the risk of SS. The overall gene-dose effect OR = 2.1 per copy of the “s” allele. COL1A1 acted synergistically with femoral neck BMD and weight in increasing prediction of wrist fracture.

McGuigan et al. examined associations with fracture and four single-nucleotide polymorphisms at the COL1A1 locus in 93 patients with vertebral fracture and 88 controls. Polymorphisms *MspI* (upstream of the COL1A1 gene Sp1 binding site), *RsaI* (in intron 5), and a *MnII* (in exon 52) were studied (8). There was an association between haplotypes Sp1 and *RsaI* polymorphisms and fracture but not the *MspI* and *MnII* polymorphisms. This association with fracture was strongest when haplotypes were grouped by Sp1 alleles. Only the Sp1 binding site polymorphism was an independent predictor of fracture (OR = 2.26, 1.1–4.7). These data suggest that it is the Sp1 polymorphism rather than other polymorphisms at the COL1A1 locus which acts as a marker for osteoporotic fractures.

More recently, McGuigan et al. reported that among 156 men and 185 women followed for a mean of 5 yr, 30 women and 9 men had fractures (9). No association with fractures and COL1A1 was seen in men but there was an association between fractures and the presence of s allele. There was an interaction with aBMD; low aBMD plus s allele conferred a 450-fold higher risk (24 fractures per 100 patient years) compared with high aBMD and SS (0.54/100 pt-yr). The results should be cautiously interpreted as 53% of the women who fractured had the SS genotype, only 3 had the ss genotype. The sample size in men precludes interpretation.

The structural basis underlying any association between fractures and a genotype is unknown. Recently, Mann et al. reported increased binding affinity of the “s” allele for Sp1 protein, and primary RNA transcripts derived from the “s” allele were three times more abundant than transcripts derived from the S allele in “Ss” heterozygotes (18). Collagen produced from osteoblasts from “Ss” heterozygotes had an increased ratio of alpha1(I) to alpha2(I) proteins and an increased ratio of COL1A1 to COL1A2 mRNA. The yield strength of bone derived from “Ss” individuals was less than derived from “SS” subjects suggesting to the authors that the COL1A1 Sp1 polymorphism is a functional genetic variant that predisposes to osteoporosis by mechanisms involving changes in bone quality.

ESTROGEN RECEPTOR AND OTHER CANDIDATE GENES

Langdahl et al. report associations between three polymorphisms (G261-C in exon 1, T-C and A-G in intron 1) of the ER alpha gene in 160 women and 30 men with spine fractures, and 124 women and 64 men with no fractures (10). The mean number of TA repeats was only slightly lower than controls even though the difference reached significance (17.3 ± 2.8 vs 18.6 ± 2.8 , respectively, $p < 0.01$); OR for fractures in individuals with 11–18 repeats was 2.6 (1.6–4.3). aBMD of the spine and hip was lower in individuals with low numbers of repeats. Mean number of TA repeats was a predictor of fractures independent of aBMD. The distribution of *BstUI*, *PvuII*, and *XbaI* polymorphisms was

similar in the fracture cases and controls leading the authors infer that a TA repeat polymorphism, but not polymorphisms in the exon 1 and intron 1, is associated with increased risk of fractures and a modest reduction in aBMD.

In a study by Becherini et al., three ERA gene polymorphisms (PVU:II, XBA:I, TA dinucleotide repeat polymorphism 5') were studied in 610 postmenopausal women (11). A difference in the number of TA repeats between women with a vertebral fracture ($n = 7$) and without was observed in women with a low number of repeats (OR 2.9, 1.6–5.7). Subjects with a low number of repeats had the lowest aBMD. The authors suggest the TA repeat polymorphism at the 5' end of the ERA gene accounts for part of the heritable component of aBMD. Given the small number of fracture cases, it is not clear whether the difference in aBMD associated with the TA repeats accounted for increased fracture risk.

Berg et al. followed subjects since 1977, 19 of 49 women sustained a fracture during 18 yr; no difference in fracture rate existed between the groups with different VDR genotypes (12). Nor was there an association detected between rate of bone loss and VDR genotypes among 77 of 118 participants followed since 1977. Houston et al. reported no VDR genotype was over represented among the 44 women with spine fractures in a sample of 171, even though individuals with bb genotype had a lower aBMD than those with BB genotype (13). Riggs et al reported no VDR genotype was over represented in 43 women with osteoporosis compared to 139 controls (14).

IL1 stimulates bone resorption while IL1 receptor antagonist (IL1ra) inhibits IL1 (15). The distribution of three IL1 gene polymorphisms (C 511 -T, G 3877 -A, C 3954 -T) did not differ among in 389 patients with spine fractures and controls. A1A1/A3 genotypes of the IL1ra polymorphism were more frequent in osteoporotics (56.2%) than controls (43.3%). The RR for fractures was 1.68 (1–2.8) in individuals with A1A1/A3 genotypes. Lumbar spine aBMD was reduced in individuals with A1A1/A3 genotypes. The authors conclude an 86-base pair repeat polymorphism in the IL1ra gene is associated with increased risk of fractures. Yamada et al report that a T to C polymorphism at nucleotide 869 of the transforming growth factor beta 1 gene (TGF beta 1) is associated with prevalent spine fractures in 118 Japanese women compared with 339 without spine fractures (16). The adjusted OR was 2.8 (1.1–7.6) for TT and TC vs CC, and 3.4 (1.2–10.1) for TT vs CC.

Thus, associations of fracture risk with genotypes are reported in the literature, most commonly for the COLIA1 and ER gene. In general, the associations reflect a relative fracture risk increase of 1.5–2.5 but the attributable risk is small and rarely transparently presented in the results section of discussed. Authors rarely, if ever, define how many *more cases* coming to fracture would be identified using the genotype in addition to risk factors such as age, aBMD, a marker of bone remodeling or presence of a fracture.

The Ambiguity of aBMD, vBMD, and Rates of Bone Loss

aBMD is a surrogate of the breaking strength of bone in vitro and is a predictor of fracture in vivo. The measurement is important in clinical practice as it provides a quantitative definition of 'osteoporosis' and is indispensable in identifying individuals at risk of fracture who should be considered for treatment. These are valid and clinically useful applications of densitometry. However, aBMD is likely to be the wrong phenotype when questions are asked about genetic factors responsible for the variance in bone *fragility* because aBMD is the summation of periosteal and endosteal (endocortical,

intracortical, trabecular) surface modeling and remodeling that has occurred during growth and aging and thus may be too ambiguous a phenotype, i.e., too insensitive, to be able to detect an association between a gene regulating a structure responsible for only a part of the deficit in aBMD (19).

A given deficit in aBMD is likely to have a heterogeneous structural basis and therefore a heterogeneous pathogenesis with many genetic factors contributing. For example, reduced aBMD may be due to reduced bone size or reduced vBMD. Reduced bone size may be due to reduced periosteal apposition during growth, aging or both. Reduced vBMD may be due to reduced accrual of cortical bone mass, trabecular numbers and thickness during growth, or due to excessive loss of these structural elements during aging. Reduced cortical and trabecular thickness may be due to excessive endosteal (endocortical, trabecular) surface resorption, itself due to increased activation frequency, an increase in the volume of bone removed by osteoclastic bone resorption or a decrease in the volume of bone replaced by osteoblastic bone formation within the BMU, or any combination of these three distinctly regulated and partly coregulated processes. How can any genetic determinant be detected when these specific multifactorial structural changes are summated into a single phenotype like aBMD?

Men lose less cortical bone than women because periosteal apposition is greater in men than in women, endocortical resorption is only slightly less in men than in women (20). The same applies to patients with fractures; the cortices may be thinner because periosteal apposition is reduced during growth or aging so that the abnormality may reside in genetic factors influencing the behavior of the periosteal apposition, not endocortical remodeling. Moreover, cortical thickness may be normal but trabecular BMD may be reduced selectively (as occurs in the early postmenopausal years when increased remodeling occurs on trabecular surfaces as trabecular bone has a greater surface to volume ratio than cortical bone). Therefore deficits in aBMD in women compared to men may have more to do with sex-specific genetic determinants of periosteal apposition than endosteal remodeling, a point lost when using aBMD as the phenotype.

Patients with fractures have smaller bones than controls (21). About 40% of the deficit in BMC and 20% of the deficit in aBMD at the spine due to the difference in bone size in cases and controls, the remainder is the result of reduced vBMD (which may be due to reduced accrual or excessive bone loss). So, looking for genes that contribute to reduced accrual or excessive loss will not be fruitful using the aBMD deficit as the phenotype. If bone size is increased in the fracture cases, aBMD may be normal obscuring any deficit in vBMD in the (larger) bone. The lack of difference in aBMD will lead the investigator to falsely infer that no reduction in accrual during growth or bone loss during aging has occurred in the fracture cases compared with controls, and so, there will be failure to seek genetic factors responsible for the larger bone size or reduced vBMD in the fracture cases.

Cortical thickness is also an ambiguous phenotype given that final cortical thickness is the net result of periosteal and endocortical modeling and remodeling throughout the whole of life. There are likely to be regulators *and* coregulators of these two surfaces working in conjunction with regulators of growth in bone length. For a given species with a given bone length, the extent of periosteal apposition and endocortical modeling and remodeling will be adapted to produce the correct long bone width and cortical thickness for biomechanical competence. Thus, the periosteal and endocortical surfaces forming the cortex must be studied separately. However, periosteal or endocortical

dimensions are dependent on age, bone age, height, weight, bone length and gender. Therefore, these covariates must be taken into consideration when variance in a trait is measured. When this is done it may be possible to quantify to genetic and environmental contributions to this variance.

Just as aBMD and cortical thickness are the net result of a great many differing regulatory processes, vBMD is also unlikely to be a suitable phenotype for the identification of genetic regulators of bone fragility because vBMD is a function of the *relative* growth of bone size and the mass accrued within it, not a function of the absolute change in bone size or bone mass. For example, in Turners syndrome, aBMD is reduced because bone size is reduced; vBMD is normal because there is a proportional reduction in size and the amount of bone accrued in the smaller bone (22). The same deficit in vBMD may be produced by entirely different mechanisms that will remain obscure unless mass and size are studied separately. For example, oophorectomy in growing rats produces a larger bone than sham operated animals but the accrual of mass is no different. The combination of a reduction in mass relative to the (larger) bone size results in reduced vBMD. Orchiectomy results in a reduction in growth in mass and size but the reduction mass is greater than the reduction in size so that vBMD is reduced. Thus, the pathogenetic mechanisms resulting in osteoporosis in the growing female and male rat differ (23).

These cell-, tissue-, surface-, region-, gender-specific structural abnormalities are the result of independent and codependent specific regulatory processes. It is difficult to envisage how an association between a specific gene or gene product and bone fragility can be detected using aBMD as a phenotypic endpoint given its structural ambiguity. This ambiguity may be partly responsible for the null observations, the inconsistent, poorly reproducible and contradictory associates found between various candidate genes and aBMD. Even when associations are reported, interpretation of the observations is not feasible because the structural basis underlying the association between aBMD and any genotype polymorphism has never been defined apart from the one example cited above (18).

The Misleading Notion of Heritability as a Constant in Nature

The study of the genetic regulation of the skeletal growth and ageing is also impeded by the common use of heritability and the mistaken belief that reporting heritability is a contribution to our understanding. The heritability of a trait is not an immutable constant in nature. If a trait has a high "heritability," this does not mean that little can be done to influence it on an individual basis. Based on twin studies, about 80% of the age-adjusted variance in aBMD is "heritable" or "genetically determined." The view is often expressed that there is, therefore, only 20% of variation "left" to modify. The implication is that environmental factors account for little variation between individuals, and therefore it is difficult to alter aBMD in an individual. The fallacy of this notion of constancy of heritability is readily seen by examining the increases in total height, sitting height and leg length during the last 50 yr. Height is a heritable trait in the sense that the cross-sectional variance is largely explained by genetic factors (24).

Variance is a mathematical measure of the scatter or the between-individual differences in a trait within a specific population. Variance is the sum of the squared distances of each point from the mean (variance in aBMD having the units g²/cm⁴). Heritability is the proportion of total variance attributable to genetic factors (25).

$$\text{Heritability (\%)} = 100 \times \text{genetic variance} / \text{total variance}$$

where total variance = genetic variance + environmental variance + measurement error.

The heritability estimate is not determined using the absolute aBMD values, but the residual values about the mean. The residuals are dependent on what variables are used to define the mean, such as age. A trait's heritability has no single unique value but varies according to which factors are taken into account in specifying the mean and what variables are used in partitioning the total variance in the specific population being studied.

The heritability estimate is applicable only to the population and environment from which the sample is drawn. Heritability is not the proportion of an individual's aBMD attributable to genetic factors. The term does not identify a percentage or a morphologically identifiable part of a trait that is genetically determined, or the proportion of disease attributable to genetic factors as often stated (26). It is incorrect to state that "80% of aBMD" is due to genes, that a given percentage of fractures are due to genetic factors, a given percentage of the population having BMD below 2.5 SD is due to genetic factors. Heritability refers to possible causes of variation—variation in a trait across a particular population.

If total variance (the denominator) increases with age due to an increase in both genetic and environmental variances, but the environmental variance increased more than the genetic variance, then heritability decreases yet the genetic component of variance has increased. If the total variance increases due to an increase in the environmental variance, the heritability will decrease despite genetic variance remaining unchanged. If measurement error is high then the heritability estimate cannot be high because the error component is contained in the denominator.

The value of quantifying the genetic and environmental components of total variance *in absolute terms* is seen when candidate genes or environmental factors are fitted to determine whether genetic or environmental factors may explain the variance. Quantifying the total variance and estimating genetic and environmental components of variance is based on principles derived by Fisher 80 yr ago. Modeling can be achieved by studying correlations between twins using the Classic Twin Model, i.e., under the assumption that MZ twin pairs share environmental factors pertinent to the trait to the same extent as do DZ twin pairs (24,25). If the MZ correlation exceeds the DZ correlation, the model must attribute the excess to the effect of shared genes. If the only reason why twins are correlated is because they share genes, the correlation between MZ pairs will be twice that between DZ pairs. If the DZ correlation is greater than one half the MZ correlation, then the amount by which it is greater must be attributed by the model to the effects of environmental factors shared by twins: the common environment variance.

GROWTH, PEAK BONE SIZE, AND PEAK VOLUMETRIC BMD

There is evidence from studies in the offspring and relatives of men and women with fractures that the deficits in aBMD are likely to have their origins at least partially in growth (27–29). There are also several animal models that support the notion that bone fragility in old age partly has its origins in growth. For example, a mouse model of growth failure and fracture has been reported as an autosomal recessive mutation, spontaneous fracture (sfx) (30). The sfx/sfx mice are phenotypically normal until after weaning, when reduced mobility and impaired growth occur. By 6 wk, IGF-1 and osteocalcin are decreased, femoral cortical thickness, diaphyseal circumference, mature surface osteoblasts, chondrocyte numbers in epiphyseal plate columns are reduced. Trabeculae in proximal tibiae, iliacs, and vertebral bodies are sparse and thin. By 78 wk, fractures of the distal femur are present. The gene responsible for this phenotype is mapped to central

chromosome 14. The study illustrates that bone fragility in old age is established during growth in sfx/sfx and that deficits in bone mass in patients with fractures may also have their origins in growth.

How does bone fragility develop during growth? What genetic mechanisms contribute to structural abnormalities responsible for bone fragility? Long bones grow in length by endochondral ossification and in width by periosteal apposition. The mass of bone in long bones may increase by increasing its length, but for a given length, the mass may increase in three ways, by increasing periosteal apposition, increasing endocortical apposition or by increasing the matrix mineral content (or density) of the bone tissue itself. There have been several advances in our understanding of the genetic regulation of growth in bone size, trabecular and cortical mass.

Matrix Mineral

In general, the matrix mineral content or amount of mineral in a volume of bone tissue remains constant from early life to old age (31), but there is variation among individuals that may have genetic basis. The mechanisms that account for difference in matrix mineral density remain obscure. There is evidence for an association between matrix mineral content and VDR genotypes. Sainz et al. reported associations between VDR and femoral cortical matrix mineral density in 100 normal prepubertal Americans of Mexican descent aged 6.7–11.7 yr (32). Care was taken to match for bone age, height, weight, body surface area, or BMI between the groups. This is critical in studies of growth because changes are very rapid. Even small differences in maturation between genotype groups (which may be nonsignificant because of small numbers) can produce large differences in structure falsely attributed to the genotype. Girls with aa and bb genotypes had a 2–3% higher matrix mineral density and 8–10% higher vertebral trabecular BMD than those with AA and BB genotypes. The 23 girls with aabb genotype had 2% higher femoral and 12% higher vertebral BMD than the 14 girls with AABB genotype. VDR alleles were not associated with vertebral or with femoral CSA or cortical bone area (measurements obtained using quantitative computed tomography). In children, accuracy of cortical density measurements is limited when the cortices are thin. Assuming no technical concerns are present, the mechanisms by which vitamin D receptor alleles affect matrix mineral density are obscure.

Cortical Thickness

Cortical thickness is determined by the relative growth of the periosteal and endocortical surfaces. The extent of periosteal expansion determines the peak diameter of the long bone. The movement of the endocortical surface relative to the periosteal surface establishes cortical thickness and the distance the cortical mass is placed from the neutral axis of the long bone, a critical determinant of bone strength. The movement of the endocortical surface is determined by the extent of endocortical bone resorption and formation. If both are equal there will be no net movement of the endocortical surface during growth and cortical thickness will be determined entirely by the extent of periosteal apposition. For any given degree of periosteal expansion, cortical thickness may be reduced if endocortical bone resorption is excessive, if bone formation is reduced, or both. Under these circumstances, as the width of the long bone increases it may develop a progressively thinner cortex.

In males there is little movement of the endocortical surface of the metacarpals so that bone resorption is matched by the same amount of apposition producing little net change in the medullary canal diameter. At weight bearing sites like the femur, there is net endocortical resorption enlarging the medullary cavity (33). At puberty in males, periosteal expansion increases, probably due to androgen and GH and IGF-1 dependent periosteal apposition. In females, estrogen inhibits periosteal expansion accounting for the smaller long bone diameter in females than males. However, estrogen, metabolites of estrogen, and perhaps progestins may inhibit endocortical bone resorption and stimulate endocortical apposition reducing the medullary diameter of the narrower long bone so that final cortical thickness is similar in men and women but constituted largely by periosteal apposition in men and by both periosteal apposition and endocortical contraction (75%:25%) in females (34).

Thus, there are surface-, gender-, and site- specific regulatory mechanisms influencing cortical mass and its geometric arrangement. What genetic factors regulate the length of the bone, its width, cortical thickness and medullary diameter? What mechanisms ensure that a bone of given length has a width that maintains bending strains below a level at which fracture will occur? If bone width is reduced, are there genetically determined mechanisms to ensure that compensatory increases will occur in cortical thickness to maintain strains within a tolerable level? If there is an abnormality in bone matrix collagen, are there genetically determined compensatory mechanisms that will modify bone size or shape to compensate for the loss of bone strength? For a given bone length, greater diameter may require a proportionately thinner cortex while a narrower bone may require a thicker cortex to reduce strains equally. There are likely to be genetically determined mechanisms that co-regulate length, width, the periosteal and endocortical surfaces to form a bone with a diameter and cortical thickness appropriate for the size and biochemical needs of that animal.

Species- and sex-specific differences in bone mass are achieved by species- and sex-specific differences in the growth of the periosteal and endocortical surfaces of long bones. For example, the SAM P6 mouse has reduced peak bone mass because the cortices are thin compared with the P2 control (35). The thinner cortices are the result of reduced endocortical bone formation during growth so that the bone has a wider medullary cavity. Whether endocortical resorption is also increased is unknown (endocortical bone resorption was not measured). Periosteal bone formation was no different to the P2 control (the P6 and P2 have similar bone diameter). The reduction in endocortical bone formation is due to a fall in the osteoblast progenitor population. Replacement of the marrow by marrow cells from P2 controls restores bone formation (36). The mechanisms responsible for the accelerated aging of the colony forming units responsible of osteoblast progenitor formation are unavailable. What ever the mechanism, the reduction in osteoblasts was confined to the endocortical surface adjacent to marrow, not the periosteal osteoblast.

Differences in the behavior of the endocortical surface is also largely responsible for differences in bone mass in C3H and B6 mice. External bone size is similar in C3H and B6 strains because periosteal bone formation is similar. The C3H mice have higher femoral and tibial BMC than the B6 because endocortical diameter is less (37). Endocortical diameter is less because endocortical bone formation normalized against total endocortical surface was greater in C3H (38). Femoral periosteal BFR/bone surface was also slightly higher in C3H than C57 in this study. The genetic basis for the strain specific differences in endocortical bone formation are not known.

There may be other mechanisms responsible for species differences in bone morphology in these animal models. Chen and Kalu also report that C3H mice have a smaller marrow area (39). Bone length, periosteal MAR and BFR did not differ by species. C3H had higher intestinal calcium absorption than B6, higher Ca uptake by duodenal cells and more occupied intestinal vitamin D receptors. The mice were fed 0.4, 0.1, or 0.02% Ca diets. Over 5 d, C3H mice maintained positive Ca absorption at all intakes, whereas B6 mice had negative Ca absorption at the two lowest Ca intakes, lower serum calcium. At 0.4% Ca, PTH was 43% higher in C3H than B6. Low Ca increased PTH in B6, not C3H. The authors suggested that in the face of a lower intestinal Ca B6 mice are more likely to rely on mobilization of Ca from bone to maintain Ca homeostasis. Endocortical bone resorption should therefore be greater in the B6 strain but this was not measured.

Linkhart et al. report that C3H mice have greater BMD and lower medullary cavity volume than B6 mice, in part due to greater bone formation and increased osteoblast progenitor cells (40). Osteoclast numbers on bone surfaces of the secondary spongiosa were twofold higher in C57 than C3H mice aged 5.5 wk. Bone marrow cells of C57 mice cocultured with Swiss-Webster osteoblasts produced more osteoclasts than C3H marrow cells. C57 marrow cell cultures formed 2.5-fold more pits in dentine slices than did C3H marrow cell cultures suggesting to the authors that differences in the osteoclast precursor population contribute to differences in BMD between these strains.

Not all studies are consistent. Turner et al also report that C3H mice had greater cortical thickness of the femur and vertebrae than B6 (41). However, the trabeculae were more highly mineralized but fewer in number in the vertebral bodies, femoral neck, and greater trochanter. Trabecular number in the C3H vertebral bodies was about half that of B6. The thick, more highly mineralized femoral cortex of C3H mice resulted in greater bending strength of the femoral diaphysis. Strength of the lumbar vertebrae was no different between strains because the thicker cortices were combined with inferior trabecular structure in the vertebrae of C3H mice.

Akhter et al. studied 16-wk-old C3H, B6, and DBA mice (42). Greater cortical area was due to greater periosteal bone formation rate. Mid-diaphyseal total femoral and tibia CSA and moment of inertia were greatest in B6, intermediate in C3H, and lowest in DBA mice. The authors suggest the specific distribution of cortical bone represents a difference in adaptation to similar loads. The size, shape, and aBMD of the bone are a result of breed-specific genetically regulated cellular mechanisms. Compared with the C3H mice, the lower aBMD in B6 mice is associated with long bones that are weaker because the larger cross-sectional area fails to compensate completely for their lower aBMD.

Heterogeneity within and between species may be the result of differences in periosteal growth. Mice made GH receptor protein (GHRP) deficient have reduced femur BMC due to a reduction in periosteal apposition rather than a reduction in endocortical remodeling (43). The defect is reversed by administration of IGF-1.

In the MOV 13 mouse, a transgenic strain carrying a provirus which prevents transcription initiation of the alpha 1(I) collagen gene, bone fragility is the result of a form of osteogenesis (44,45). Heterozygotes for the null mutation survive into adulthood and their fibroblasts make less collagen type I. In this mouse there is an adaptation that compensates for the bone fragility; bending strength is increased in this animal by compensatory periosteal apposition. This study suggests that, in this model of osteogenesis, continued periosteal bone formation compensated for the marked reduction in type I collagen synthesis, maintaining bending strength relative to wild type controls.

Hankenson et al report that thrombospondin 2 (TSP2)-null mice have abnormal collagen fibrils, increases in ligamentous laxity, but increased cortical density due to increased endosteal bone formation (46). Mechanical properties of femurs were normal suggesting structural changes occurred to compensate from the qualitative defect in bone. Marrow stromal cells (MSCs) were present in increased numbers in a colony forming unit (CFU) assay and showed an increased rate of proliferation in vitro suggesting TSP2 regulates the proliferation of osteoblast progenitors and that in its absence endosteal bone formation is increased. These two examples suggest that there are pathogenetic mechanisms that allow the skeleton to adapt so that it can serve its biomechanical function. What genetic factors are involved in the compensatory response?

Trabecular Number and Thickness

Trabecular vBMD is determined by the number and thickness of trabeculae. Trabecular numbers are formed at the growth plate before puberty. Chung et al examined the mechanism by which chondrogenesis proceeds to osteogenesis (47). Indian hedgehog (Ihh) couples chondrogenesis to osteogenesis in endochondral bone development. PTHrP, synthesized in the periarticular growth plate, regulates the site at which chondrocyte hypertrophy occurs. The investigators compared PTH/PTHrP receptor(–/–)/wild-type (PPR–/–/wild-type) chimeric mice with Ihh(–/–);PPR–/–/wild-type chimeric and Ihh(–/–)/wild-type chimeric mice. Ihh, synthesized by prehypertrophic and hypertrophic chondrocytes regulates the site of hypertrophic differentiation by signaling to the periarticular growth plate and determines the site of bone collar formation in the perichondrium.

Calvi et al. report that the PTH/PTHrP receptor participates in skeletal development. PTH targets most of its actions in bone to the osteoblast lineage (48). PTH stimulates osteoclastogenesis through activation of osteoblastic cells. To assess the role of the PTH/PTH-related protein receptor (PPR), transgenic mice that express, in cells of the osteoblastic lineage, one of the constitutively active receptors in Jansen's metaphyseal chondrodysplasia show increased osteoblastic function in trabecular and endosteal compartments, and decreased function in the periosteum. Osteoblast precursors and mature osteoblasts were increased in trabecular bone. Osteoblastic expression of the constitutively active PPR induced an increase in osteoclast number in both trabecular and compact bone. Trabecular bone volume increased and cortical bone thickness of the long bones decreased.

The formation of the trabecular numbers and their thickness depends on the rate of resorption as well as formation. If resorption is decreased osteopetrosis may result. Gowen et al. report that mice deficient in the cathepsin K gene had, at age 2 mo, osteopetrosis of long bones and vertebrae with increased trabecular number and thickness at the primary spongiosa and metaphysis and increased cortical thickness (49). Epiphyseal growth plates showed diminished osteoclast activity at the zone of cartilage calcification and primary spongiosa. Chondrocyte differentiation was normal. Analysis of resorption sites suggested normal demineralization via vacuolar ATPase activity, but reduced resorption and endocytosis of bone matrix. Cathepsin-K deficient osteoclasts are unable adequately to remove demineralized bone, an effect consistent with the function of cathepsin K as a matrix degrading proteinase.

De Luca et al. studied the effects of bone morphogenetic proteins (BMPs) on embryonic skeletal development (50). BMP-2, expressed in the growth plate, regulates growth plate chondrogenesis and longitudinal bone growth. Fetal rat metatarsal bones were cultured for 3 d in the presence of recombinant BMP-2. BMP-2 accelerated of metatarsal

longitudinal growth by stimulating chondrocyte proliferation in the epiphyseal zone of the growth plate. BMP-2 also increased chondrocyte hypertrophy and cartilage matrix synthesis. These BMP-2-mediated stimulatory effects were reversed by recombinant human Noggin, a glycoprotein that blocks BMP-2 action. In the absence of exogenous BMP-2, Noggin inhibited metatarsal longitudinal growth, chondrocyte proliferation, and chondrocyte hypertrophy, which suggests that endogenous BMPs stimulate longitudinal bone growth and chondrogenesis. BMP-2 accelerates longitudinal bone growth by stimulating growth plate chondrocyte proliferation and chondrocyte hypertrophy.

FGF-2, a modulator of cartilage and bone growth and differentiation, is expressed and regulated in osteoblastic cells (51). Mice with a disruption of the FGF2 gene had trabecular bone architecture of the femoral metaphysis were reduced and many of the connecting rods were lost in FGF2^{-/-} mice. A decrease in trabecular bone volume, mineral apposition, and bone formation rates was observed.

The dopamine transporter (DAT) may control the activity of released DA by rapid uptake of the neurotransmitter into presynaptic terminals. Mice homozygous for deletion of the DAT gene (DAT^{-/-}) had reduced femur size, cortical thickness, ash content and strength (52). Cancellous bone volume of the proximal tibial metaphysis was lower with reduction in trabecular thickness. Vertebral cancellous bone volume was lower due to reduced trabecular number. Thus, deletion of the DAT gene results in deficiencies in skeletal structure and integrity.

Trabecular numbers do not increase during growth but remain constant (53). Indeed, trabecular vBMD remains constant during prepubertal growth (54). In other words, as the vertebral body increases in size, the number of trabeculae remains constant but the existing trabeculae increase in length and width as the vertebral body increases in size, but this increase in the external size and the increase in length and width of existing trabeculae is proportional. The importance of the constancy of vBMD in early life is threefold. (i) the variance in trabecular BMD must be determined early in life, or prenatally. Therefore, an individual with trabecular vBMD at the 5th percentile at the age of 2 yr is likely to also have trabecular vBMD at the 5th percentile at maturity provided that this trait "tracks." There is evidence for tracking of this trait. What are the genetic factors that determine trabecular number? (ii) As vBMD is a function of the increase in size and mass within its periosteal envelope and as vBMD remains constant and at a given percentile during prepubertal life, there are likely to be genetic factors that regulate and co-regulate the growth in external bone size and the mass within it. (iii) As there is a large variance in vBMD, then for a given bone size, the amount of accrual in one bone must be greater than in another of the same size to produce this variance. That is, some individuals with a given bone size must be accruing more bone per unit external volume of bone to place vBMD at the 95th percentile, while others must accrue less, placing their vBMD at the 5th percentile.

At puberty, trabecular BMD increases, and increases similarly in males and females of a given ethnic group. The increase in trabecular BMD is the result of further thickening of existing trabeculae rather than in numbers of trabeculae. African Americans and Caucasians have the same trabecular number and thickness before puberty but the increase in trabecular BMD at puberty is greater in African Americans than Caucasians (55). Therefore, there are race specific factors regulating pubertal increase in trabecular BMD but the increase is not sex specific, being the same in males and females of a given race. What genetic factors determine the variance in trabecular thickness and the race specific variability in trabecular thickness?

The genetic factors conferring a higher or lower vBMD are unknown but this information is important because the position of an individual's peak vBMD in young adulthood determines the vBMD for many years later. Prepubertal growth is sex hormone and GH/IGF-1 dependent in males and females. Whether some individuals are more sensitive to these regulators of growth and so develop greater trabecular numbers or thickness (and so have vBMD at the 95th percentile) during prenatal, postnatal or peripubertal growth is not known.

Rosen et al. reported differences in femoral vBMD in C3H than B6 mouse strains (56). Serum IGF-I was over 35% higher in C3H than B6. F1 progeny had IGF-1 and femoral BMD levels intermediate between the parental strains. F2 progeny with highest BMD had highest IGF-1 while skeletal IGF-1 (calvaria, tibia, femora) was ~30% higher in C3H than B6 mice. The authors inferred that the difference in vBMD between strains may be related to systemic and skeletal IGF-I synthesis. Whether the greater endocortical bone formation in the C3H is explained by greater IGF-1 is unclear.

The Regulation of Bone Growth in Human Subjects

Most studies in humans concerning the genetic regulation of growth use aBMD as the phenotype so that the structural basis of any genotype specific differences in aBMD is obscure. In addition, the results must be carefully examined before inferences made by the authors is accepted as there are often small differences in chronological or maturational age between the genotypes. Because of the rapidity of growth, even a few months difference in maturational development can produce large differences in bone size and bone mass which are attributed to the genotype rather than this design flaw.

In a study of 423 infants, Suarez et al. reported that girls with the BB genotype of the vitamin D receptor had greater weight, length, and surface area at 2 yr than those with the bb genotype (57). Boys with BB genotype had lower weight and surface area at 2 yr than bb and Bb boys. Thus, gender differences were only observed in Bb and bb groups. In a longitudinal analysis of 145 full term babies, gender differences in weight, length, and surface area existed in the bb infants. Keen et al. reported an association between VDR genotypes and weight at one year in 66 adult women (58). The tt group had 7% greater weight than the TT. However, VDR genotypes were not associated with aBMD at the spine or proximal femur, height, or weight in adulthood. Gunnes et al reported changes in aBMD over about 4 yr in 273 healthy boys and girls aged 8.2–16.5 yr were not associated with VDR genotype (59). The rationale underlying the search for differences in body weight by genotype and the biological significance of these observations is obscure.

Racial differences in aBMD have been attributed to genotypic differences. Harris et al. determined the *FokI* genotype of the vitamin D receptor gene in 72 black and 82 white premenopausal women aged 20–40 yr (60). Four percent of blacks and 18% of whites were homozygous for ff, and 65% and 37% were homozygous for FF, respectively. In whites, women with the ff genotype had 4.3% lower total body aBMD and 12.1% lower femoral neck aBMD than FF. Spine aBMD did not differ by genotype. Adjustment for the *FokI* genotype reduced the racial difference in femoral neck aBMD by 35%. The authors suggest that the polymorphism appears to influence peak aBMD and differences in its distribution may explain racial variations in aBMD. However, associations were not examined after taking differences in body size into account, and the morphological basis underlying the genotypic and racial differences in aBMD were not defined. Gross

et al reported that women with the ff genotype had 12.8% lower spine aBMD than the FF group (61). This was not seen in the study by Harris et al. (60). Follow up over 2 yr showed the decrease in femoral neck aBMD was greater in women with ff than with FF (-4.7 vs -0.5% , $p = 0.005$), yet lumbar spine aBMD was reduced but rates of loss were greater at the femoral neck.

Arai et al. report a thymidine-cytosine (T-C) transition polymorphism at the translation initiation codon of the VDR gene in 239 Japanese females (62). Among 110 premenopausal women, spine aBMD was 12% higher for mm than MM homozygotes. Cloned DNA from the ATG (M allele) variant yielded a 50 kD protein (MP), and that from the ACG (m allele) variant a 49.5 kD protein (mP) in transfected COS-7 cells. Vitamin D dependent activation of a luciferase reporter construct in transfected HeLa cells was increased 19.5-, 11.2-, and 15.5-fold in cells expressing, respectively, mP, MP, and both mP and MP. The extent of expression of mP was ~20% less than that of MP in vitro and in transfected HeLa cells. The authors conclude that the T-C polymorphism results in synthesis of a smaller protein with increased biologic activity associated with increased aBMD. By contrast, Eccleshall et al. report that the start codon polymorphism (SCP) of the VDR gene, examined in 174 premenopausal women aged 31–56 yr, was unrelated to BsmI genotype, BMD, calcium, PTH, or vitamin D levels (63). NTX was 33.5% higher in ff than FF women, suggestive of a difference in bone resorption rate, but no other biochemical markers differed. Inferences are difficult to make when results are so contradictory.

Mizunuma et al. reported the association of ER polymorphisms, lumbar aBMD and bone turnover in 173 pre- and postmenopausal women (64). Women with Xx genotype had higher aBMD than those with xx genotype. The authors suggested that the Xx genotype may be associated with greater accretion of bone during young adulthood.

As an association between a 3' UTR polymorphism of the IL-6 gene and BMD is reported, Takacs et al searched for linkage between this polymorphism and peak aBMD in 812 healthy premenopausal sibpairs but found no evidence for linkage between the IL-6 gene locus and aBMD in Caucasians or African Americans (65). Lorentzon et al. examined a G/C polymorphism in position -174 of the IL-6 gene promoter in 90 boys, aged ~17 yr. Boys with the CC genotype had ~7% higher aBMD at several sites than GG counterparts but did not predict the increase in aBMD that occurred during 2 yr followup (66).

AGING AND CHANGES IN BONE SIZE AND VOLUMETRIC BMD

Menopause- and Age-Related Bone “Loss” and Genotypes

Just as the vagaries of aBMD and vBMD are likely to be an impediment to understanding the genetic causes of bone fragility, the lack of specificity in characterizing bone “loss” into its distinct morphological components is likely to be an impediment in the search for genetic factors contributing to bone “loss.” Bone “loss” is not just resorptive ‘removal’ of bone, it is also failure of bone formation. Bone “loss” during aging is also the net result of the amount of bone resorbed on the endosteal (intracortical, endocortical, trabecular) surfaces and the amount formed on the periosteal surface. The net amount of bone resorbed on the endosteal surfaces is a function of the imbalance between the absolute volume of bone resorbed and formed at the BMU, and the rate of bone remodeling (activation frequency).

Thus, examining the rate of bone loss using densitometry, which integrates all of these changes, to identify genetic factors accounting for the variance in net bone loss is fraught

with problems. Bone loss probably needs to be broken down into less ambiguous components for successful identification of its regulators. Two individuals may lose exactly the same percentage of the same baseline aBMD by entirely different mechanisms. The imbalance at the BMU may be the same, but due to reduced bone formation in some patients, increased bone resorption in others. The imbalance at the BMU may be less in one individual but remodeling rate may be higher. Both remodeling rate and imbalance in the BMU may be identical but one subject may have greater periosteal apposition than other so it appears (using densitometry) that one has lost more bone than another. Genetic factors contributing to reduced bone formation or increased bone resorption in the BMU, activation frequency, periosteal apposition will be obscure unless the heterogeneity of bone loss between individuals, and in an individual at different times of life, is recognized and adequately measured.

Bone loss is greater in women than men because men have greater absolute gain in bone by periosteal apposition, the absolute amount of bone resorbed on the endosteal surfaces do not differ very much in men and women (20). Periosteal apposition during ageing differs by region, gender, race, and perhaps according to biomechanical factors. These components of bone modeling and remodeling have rarely been defined in terms of their age-, gender-, and menopause- specific means and variances so that no information is available regarding the roles of candidate genes in determining the variance for each of these traits.

ESTROGEN RECEPTOR

Bone loss after menopause is estrogen dependent. In the elderly, secondary hyperparathyroidism associated with calcium malabsorption may partly contribute to bone loss by increasing cortical bone remodeling. Perhaps polymorphisms of the ER gene or PTH receptor gene may be associated with activation frequency at differing stages of the aging process. Bagger et al. investigated the possible relation of the *PvuII* and *XbaI*RFLPs of the ER gene and aBMD in a cross-sectional study of 499 postmenopausal women and prospective study of 101 postmenopausal women followed for 18 yr (67). Postmenopausal bone loss at the hip and spine was determined over 6 yr and bone loss in the lower forearm over 18 yr. No effect of the ER genotypes on rates of loss was found.

On the other hand, Salmone et al. reported an association between ER genotypes and aBMD before and after a 5-yr HRT in a placebo-controlled, population-based study of 322 early postmenopausal women (68). At baseline, no differences in the lumbar or femoral neck aBMD were present between the ER *PvuII* groups. In the non-HRT-group, lumbar spine aBMD decreased more in the PP (6.4%) and Pp (5.2%) than pp (2.9%) ($p = 0.002$) leading the authors to infer that subjects with the ER *PvuII* genotypes PP and Pp may have a greater risk of bone loss after menopause than those with the pp genotype and that they may preferentially derive benefit from HRT. No changes occurred at the femoral neck.

Han et al. investigated the presence of three restriction fragment length polymorphisms at the ER gene locus in 248 healthy postmenopausal Korean women aged 41–68 yr (69). No relationship was present between genotypes and spine aBMD. Kobayashi et al. investigated 238 postmenopausal Japanese aged 45–91 yr (70). aBMD at lumbar spine and total body were higher in homozygotes for the Px haplotype (PPxx), to heterozygotes (PPXx, Ppxx), than those lacking the Px haplotype (PPXX, PpXX, ppXx, and ppxx) leading the authors to conclude that variation in ER gene partly explains low

aBMD in postmenopausal Japanese women. All these analyses are unlikely to be preplanned as there is no physiological basis for a hypothesis-driven examination the various haplotypes. These are likely to be posthoc analyses and confounders may be present that are responsible for the differences.

Willing et al. examined the association between aBMD and changes in aBMD over 3 yr in about 250 women and polymorphisms for the VDR, ER, type 1 collagen, and other genetic markers (71). ER polymorphisms predicted aBMD but not changes in aBMD. VDR polymorphisms were not associated with BMD or changes in aBMD. Gene-gene interactions were reported as certain combinations of bb and (–/–) *PvuII* had higher and BB and (–/–) *PvuII* had lower aBMD.

Type I Collagen and Other Candidate Genes

In some studies crosssectional data are used and the diminution across age is referred to as a “rate” of loss. Uitterlinden et al. report that 526 women with the Ss genotype had 2% lower femoral neck and lumbar spine aBMD, while 58 with the ss genotype had 4% lower femoral neck and 6% lower spine aBMD compared 2294 women with the SS genotype of COLIA1 (72). Differences were smaller (and not reported) but still significant after adjusting for the lower body weight in the ss group. The genotype explained 0.2–0.4% of the variance in aBMD. The authors found no aBMD differences in the 55–65-yr olds according to genotype. Among the 75–80 year-olds the 91 Ss had 5% lower femoral neck aBMD ($p = 0.03$) and 3% lower lumbar spine aBMD (NS) than the 205 SS. The 7 ss group had 12% lower at the femoral neck and 20% lower at the spine ($p = 0.04$ and 0.004, respectively). These cross-sectional data do not provide compelling evidence for more rapid bone “loss” in the ss or Ss genotype and it is difficult to conclude, as the authors do, that genotyping at this site may complement information for fracture risk gained from the aBMD measurement. If the question is whether bone loss is more rapid in one genotype then subjects should be stratified by genotype with prior matching by age, baseline aBMD, menopausal status, body weight and other factors that may influence the rate of loss.

Harris et al. examined associations of the COLIA1 genotype with 5-yr rates of change in BMD in 243 men and women aged 65 yr and older (73). The distribution of the genotypes was 155, 79, and 9 in the SS, Ss, and ss. Baseline aBMD did not differ by genotype site. The 5-yr percent changes in aBMD at the total body was about –0.3, –0.6, and –3%. Results at the femoral neck were similar, but not significant. There was no effect of genotype on change in spine aBMD. Caution is needed in the integration of these results because of the small number of subjects in the ss genotype. Heegaard et al. report no association between bone loss during 18 yr follow-up of 133 postmenopausal women and COLIA1 Sp1 polymorphisms. There was no aBMD difference between the genotypes (74). Women with the Ss or ss genotypes had higher aBMD at the distal forearm than women with the SS genotype. The levels of osteocalcin and urinary collagen type I degradation products were not associated with the COLIA1 Sp1 polymorphism.

Several studies suggest an association between rates of bone loss and VDR genotypes. Krall et al. reported that 229 healthy postmenopausal women at 0.5–37 yr postmenopause had greater rates of bone loss over two years in the BB group at the femoral neck, radius, and spine (77). Yamagata et al. reported that seven women with the BB genotype lost bone more rapidly during 12 mo than did the other two genotype groups (78). Ferrari et al. reported that among 72 subjects aged around 74 yr, bone loss at the spine of over 0.5%

per year was found in 7 of 9 subjects with the BB allele, in 15 of 37 with Bb, and in 8 of 26 with bb ($p < 0.01$) (79). The annual rate of change was greater in BB subjects (-2.3%) than in bb (0.7%) and Bb (1%). There were no differences in bone loss at the proximal femoral BMD between the groups.

Garnero et al. studied 189 healthy premenopausal women aged 31–57 yr (75). No differences in bone formation (osteocalcin, bone alkaline phosphatase, PICP), bone resorption (CrossLaps, NTX), or aBMD were found between the three VDR genotypes. These polymorphisms were concluded not to be predictive of bone turnover or BMD in these premenopausal women. Garnero et al. also reported no relation of VDR genotype and BMD at spine, hip, forearm, or whole body, with rate of bone loss, or with markers of bone formation or bone resorption 268 postmenopausal women aged 50–70 yr (76). There were no differences in age, years since menopause, body mass index, or dietary calcium intake that may obscure any associations. Rates of bone loss measured over 2 yr were significant but did not differ between the genotypes. Analyses confined to 128 women within 10 yr of menopause did not identify more rapid bone loss in any of the genotypes.

As bone loss in the elderly may be partly explained by secondary hyperparathyroidism due to calcium malabsorption, more rapid bone loss in one genotype may be the result of an association between reduced calcium absorption and one of the VDR genotypes. Gennari et al. examined this possibility in 120 postmenopausal women aged 52–75 yr (81). VDR genotypes showed no association with femoral neck or lumbar aBMD. Intestinal calcium absorption, measured with SrCl_2 , was lower in BB than bb, in tt than TT, and in AABbTt than aabbTT or AaBbTt genotypes. PTH, alkaline phosphatase and vitamin D metabolites did not differ by genotype. Zmuda et al. studied 156 African-American women aged 65 yr and older and reported that fractional Ca-45 absorption was 14% lower in women with BB than bb genotype ($p = 0.08$) but no association between VDR genotypes, aBMD or bone turnover markers was observed (82). In another study, women with the BB and bb genotypes had similar fractional calcium absorption on a high calcium intake. However, women with the BB genotype had a lower fractional calcium absorption and a reduced incremental rise in intestinal calcium absorption in response to calcium restriction than those with the bb genotype (83). Kinyamu et al. reported no differences in intestinal VDR protein concentration, calcium absorption, or serum 1,25 dihydroxyvitamin D among 92 Caucasian women aged 25–83 yr as a group or in the 25–35-yr or 65–83-yr old women (84). Francis et al. report that no association between fractional calcium absorption or aBMD and VDR genotypes in 20 men aged around 60 yr with vertebral crush fractures and 28 controls (85). Barger-Lux et al. found no relationship between the allele and the receptor density in duodenal mucosa among 35 premenopausal women (86).

Salamone et al. studied the rate of change in aBMD among 392 healthy, pre-, peri-, and postmenopausal white women during 2.5 yr (80). Spine bone loss was greater in peri- and postmenopausal women with than without an APOE4 allele with the allele explaining 6.1% of the bone loss. Among non-HRT users, there was a twofold higher rate of spine bone loss in women with than without an APOE4 allele.

BMD Responses to Intervention and Genotypes

There have been several studies claiming that a response to drug therapy is greater in one genotype than another. These studies should be viewed with scepticism as none have been randomized placebo controlled trials conducted with prior stratification according to geno-

type followed by random allotment of placebo or drug within each genotype. Most studies have been done in trials conducted years before with blood sampling for genotype classification done more recently. Obviously, the genotype will not have changed, but as there has been no stratification, it is possible that there are differences in factors other than the genotype that may explain a difference in response in BMD or a biochemical marker. Slight differences in the groups in age, years postmenopause, dietary calcium intake, baseline aBMD may not be statistically significant (conveniently assured by small sample sizes) but the difference may be enough to either produce a difference in one genotype (producing a type 1 error) or obscure a difference (producing a type 2 error)

Graafmans et al. reported the results of a two year study in 81 women aged about 70 yr receiving placebo or vitamin D (400 IU daily). Increased aBMD in the vitamin D group relative to placebo group was 4.4% in the BB genotype ($p = 0.04$), 4.2% Bb genotype, and -0.3% in the bb genotype group (87). It is not clear from the paper whether the *changes* in the BB differed from the *changes* in the bb. Tajima et al. reported that functional difference between the VDR *FokI* genotypes in response to training (88). However, the duration of training was one month with only 10 subjects in the FF and 10 in the Ff or ff genotypes make the interpretation of any changes difficult.

Responses to treatment were compared in TGF- β 1 genotypes in 363 postmenopausal Japanese women (130 untreated), 117 (1 α hydroxyvitamin D or 1,25 dihydroxyvitamin D) and 116 (HRT) (89). In controls, the rate of bone loss decreased according to the TT, TC, CC, with a difference detected between the CC and TT genotypes. The positive response of spine aBMD to HRT increased according to the ranking TT, TC, CC. Individuals with the CC genotype responded to vitamin D with a 1.6% annual increase in spine BMD, whereas those with the TT or TC genotypes lost bone. The authors infer that these results suggest that TGF- β 1 genotype is associated with the rate of bone loss and the response to active vitamin D.

In a cross-sectional study, 425 ambulatory postmenopausal women 42–85 yr old, were genotyped for *BsmI* polymorphism at the vitamin D receptor (VDR) gene and the *PvuII* polymorphism in the estrogen receptor (ESR1) gene (90). To investigate the interaction between HRT and receptor genotypes in an effect on heel stiffness index (SI). A two-locus genotype (VDR-bb/ESR-PP) present in 9.5% of women explained over 30% of the total HRT-related heel SI difference in the sample. Women bearing this combined VDR/ESR1 genotype who received HRT for more than 5 yr had a 21% (1.25 SD) greater heel SI than those bearing the same genotype but who received HRT for <5 yr. This may translate into a two- to threefold difference in the risk of fracture suggesting to the authors that QUS of the heel in postmenopausal women taking HRT is affected by variation in VDR and ESR1 loci, jointly.

Han et al. found no association between polymorphisms of the ER gene and the response to HRT in terms of aBMD mean decrements in biochemical markers in 248 healthy postmenopausal Korean women (69). Marc et al. reported that 24 postmenopausal women aged 56–73 yr with osteoporosis received cyclic etidronate (400 mg/d) and calcium (1000 mg/d) for 1 yr (91). Lumbar aBMD increased 7.3% in BB and 7.0% in Bb groups, and 2.5% in bb. Osteocalcin decreased 54.2% in bb compared to 32.4% in BB women. The authors conclude that response to bisphosphonate therapy combined with calcium supplementation is modified by *BsmI* polymorphism of the VDR receptor.

Genetic and Environmental Components of Variance in aBMD

There are no studies examining the age- and sex-specific variance in height, sitting height, leg periosteal or endosteal surfaces, bone length, width, cortical thickness or any other specific trait. All studies, few as they are, are confined to aBMD as the phenotype. Smith et al. reported that total variance in aBMD and bone width but not height, increased with advancing age and was higher in adult twins compared with juvenile twins (1). Hopper et al. reported that total variance in aBMD increased during pubertal growth and most of the variance is attributable to unmeasured genetic factors (25). The genetic variance in twins aged 13–17 yr is less than in twins aged 10–13 yr, while the common environmental variance was higher in the 13- to 17-yr old group. The factors responsible for the common environmental variance are unknown. The covariance, a measure of resemblance, increased in MZ twins as did the total variance, while the covariance of DZ twins increased during adolescence but then decreased in early adulthood. Identical twins are more likely to choose a similar lifestyle in adulthood so remain similar, while DZ twins are more likely to pursue a more independent lifestyle and consequently become increasingly dissimilar.

Common Genetic Determinants of Bone Mass and Body Composition

In elderly twins, Flicker et al. reported that the genetic variance accounts for most of the total variance in aBMD, but a common environmental component of variance was identified (92). The absolute and relative sizes of the genetic and environmental components of variance varied from site to site. Seeman et al. reported that the genetic variance accounted for 65% of the total variance in aBMD at the femoral neck in adult twins (93). When adjusted for age and lean mass, the genetic variance decreased by 16% at the femoral neck but the common environmental variance remained unchanged. At the lumbar spine, neither the genetic nor common environmental variance changed after adjusting for age and lean mass. This is consistent with cross-sectional and within-pair analyses which showed that aBMD is associated with lean mass, and more so at the femoral sites. A reduction in variance of proximal femoral aBMD, but not spine aBMD, after adjusting for lean mass could suggest that exercise may be important at the former site. If this was the case, however, then the fall in variance on adjusting for lean mass should have been in the environmental component (although genetic factors may also explain variation in exercise). The observed fall in the genetic component suggests that there are genetic determinants of both aBMD and lean mass. That is, there are genes which influence variation in both aBMD and lean mass.

To address this, the covariance between lean mass and bone mass was examined by comparing the cross-trait correlations in an individual and between co-twins. The cross-trait correlation in self, i.e., between an individual's total body lean mass and her own femoral neck aBMD, was about 0.25. The cross-twin cross-trait correlation (lean mass in twin 1 vs areal BMD in twin 2) was similar to the cross trait correlation in self for the MZ twins and greater than in DZ twins. When height was taken into account, the cross-trait correlations in self decreased, and the cross-trait cross-twin correlations were not different between MZ and DZ pairs, suggesting that genes regulating size are involved in the genes which are common to areal BMD and lean mass (93).

Arden and Spector, in a study of 227 pairs of monozygous and 126 pairs of dizygous female twins aged 45–70 yr, reported higher intraclass correlations for muscle strength and mass in monozygous than dizygous twins (0.69 ± 0.03 vs 0.41 ± 0.07 for lean body mass, 0.49 ± 0.05 vs 0.31 ± 0.08 for grip strength, 0.44 ± 0.60 vs 0.14 ± 0.10 for leg extensor

strength, after adjusting for age, height, and weight) (94). Heritability estimates demonstrated a genetic component to lean body mass, grip strength, and leg extensor strength of 0.46, 0.30, and 0.52, respectively. However, the total additive genetic influence of aBMD was reduced on adjusting for muscle variables suggesting that there may be common genetic factors regulating muscle and bone mass. The authors also reported an independent association between muscle strength and bone mass after adjusting for lean mass.

Nguyen et al., in a study of 57 monozygotic and 55 dizygotic female twin pairs aged 52.8 ± 13 yr, reported that intrapair differences in lean mass were associated with intrapair differences in femoral neck aBMD, fat mass was associated with TBBMD, while both lean and fat mass were independent determinants of lumbar aBMD (95). By contrast to the above studies, these investigators reported that the genetic correlation of lean mass and BMD was not significant and that the association between lean and fat mass was attributable mainly to environmental factors. The authors conclude that lean and fat mass and BMD are under strong genetic regulation, but the associations between them are mediated largely via environmental influences. The reasons for the disparate observations are not apparent.

Guesens et al. reported an association between muscle strength and the VDR genotypes. The investigators found no association between BMD at femoral neck, lumbar spine, and proximal forearm and VDR genotypes in 501 healthy women aged above 70 yr (96). However, in a posthoc analysis, in non-obese women, femoral neck BMD was 5% higher in women with bb than BB genotypes (z scores were -0.35 ± 0.13 for BB, -0.09 ± 0.07 for Bb, and -0.04 ± 0.09 for bb). Women with BB genotype had 23% lower quadriceps strength than those with bb genotype, which remained significant after adjusting for age, calcium intake, and femoral neck aBMD. The reason for adjusting for aBMD is not apparent. Whether there were differences in exercise activity or muscle size that may account for the muscle strength differences was not examined. After correction for quadriceps strength, no difference in femoral neck aBMD was found between the VDR types in nonobese women. From this data, the authors infer that an association exists between muscle strength and an allelic variant at the VDR locus in non-obese elderly women, which could contribute to the association between VDR polymorphism and femoral neck aBMD.

SUMMARY AND CONCLUSION

The questions and methodological problems that need close attention in the study of the genetics of bone fragility are not very different from the study of any risk factor for bone fragility. What are the risk factors (genes) that contribute to bone fragility? How do the risk factors produce bone fragility? How large are the effects, how common are they? What proportion of the fractures in the community are explained by the risk factors (genes)? When there is an association between a genotype and fractures, aBMD, rate of bone loss, a response to drug therapy, what structural abnormality explains the fragility, what cellular mechanism accounts for the more rapid net loss of bone or greater response to drugs has never been defined in any study. No gene, gene product, gene polymorphism has been reproducibly shown to account for a given proportion of the variance in aBMD. The data concerning candidate markers such as polymorphisms of the vitamin D receptor, estrogen receptor, and type 1 collagen genes is inconsistent, partly because of the questionable value of the phenotype being studied, the use of genetic markers of uncertain biological function, flaws in study design such as small sample sizes, failure to account for confounding, lack of stratification and/or randomization prior to intervention,

reliance on statistical adjustment rather than study design, and the use of posthoc analyses to infer causation. Heritability estimates, used as the measure of genetic effect, provide a measure of the proportion of the age-specific population variance attributable to genetic factors but little if any clinically useful information. These flaws need to be addressed. Distinct morphological phenotypes (height, bone length, bone width, endocortical width, cortical thickness, trabecular number, thickness) should be carefully quantified according to their age-, gender-, and race-specific means and variances. Genetic and environmental factors should be sought that explain variance in these and other traits. Expressing variance in absolute terms and fitting candidate genetic and environmental factors that may explain variance is hypothesis generating. Controlled randomized trials with prior stratification by genotype are needed to identify genotype-specific effects. The null hypothesis states that no biologically meaningful effect exists between genotypes, skeletal growth, aging and effects of treatment. This hypothesis cannot be rejected.

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3

Peak Bone Mass Acquisition

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DEFINITION AND IMPORTANCE OF PEAK BONE MASS

Peak bone mass (PBM) corresponds to the amount of bony tissue present at the end of skeletal maturation. It is a major determinant of the risk of fractures, such as those observed at the radial, vertebral, or femoral sites in osteoporotic patients. From epidemiological studies it can be assumed that an increase by 10%, i.e., by approx 1 standard deviation (SD) of PBM in the female population decreases the risk of fracture by 50%. Hence the interest of exploring ways of increasing PBM in the primary prevention of osteoporosis. Bone mineral accumulation from infancy to postpuberty is a complex process. It can now be better appreciated with the availability of noninvasive techniques able to precisely measure areal (a) or volumetric (v) bone mineral density (BMD) at several sites of the skeleton by either dual X-ray absorptiometry (DXA) or quantitative computed tomography (QCT). These techniques also allow one to capture part of the change in the macroarchitecture or geometry of the bones which along with the mineral mass strongly influences the resistance to the mechanical strain. This chapter attempts to summarize some of the knowledge that has accrued over the past few years on the characteristics of normal bone mass development from infancy to the end of the skeleton maturation.

CHARACTERISTICS OF PEAK BONE MASS ACQUISITION

Measurement of Bone Mass Development

Most information on the characteristics of skeletal growth during childhood and adolescence has been obtained thanks to the availability of noninvasive techniques allowing one to quantify with great precision and accuracy bone mass at various sites of the skeleton (1). The bone mass of a part of the skeleton is directly dependent upon both

its volume or size and the density of the mineralized tissue contained within its periosteal envelope. The mean volumetric mineral density of bony tissue (BMD in g of hydroxyapatite per cm^3) can be determined noninvasively by quantitative computed tomography (QCT). The technique of either single or dual X-ray (SXA, DXA) absorptiometry provides measurement of the so-called “areal” or “surface” bone mineral density (BMD in g of hydroxyapatite per cm^2). The values generated by this technique are directly dependent upon both the size and the integrated mineral density of the scanned skeletal tissue. This second variable is made of several components including the cortical thickness, the number and thickness of the trabeculae and the “true” mineral density corresponding to the amount of hydroxyapatite per unit volume of the bone organic matrix. The term bone mineral *density* without the additional “areal” qualification has been widely used with the general understanding that neither the SXA nor the DXA techniques provides a measurement of volumetric density. This notion, which should be obvious to bone biologists using DXA technology in either experimental or clinical settings, has not always been fully appreciated leading to mis- or overinterpretation of the data generated by this noninvasive technology (2). Therefore, it has to be reemphasized that aBMD is the summation of several structural components which may evolve differently in response to genetic and environmental factors. Nevertheless, areal BMD remains of clinical relevance in the context of osteoporosis. Indeed, the values of areal BMD have been shown to be directly related to bone strength, i.e. to the resistance of the skeleton to mechanical stress both in vivo and in vitro (for review, *see refs. 3 and 4*). Thus, there is an inverse relationship between areal BMD values and the prevalence of osteoporotic fractures (5).

At the spinal level, the total mineral content (BMC in g of hydroxyapatite) of the vertebrae, including the posterior arch, can be measured using the classical antero-posterior (frontal) projection. The BMC and the derived areal BMD of the vertebral body “isolated” from the vertebral arch can also be obtained by using DXA in the lateral (sagittal) projection (6,7). The so-called bone mineral “apparent” density (BMAD in g/cm^3) is an indirect and rather imprecise estimate of the volumetric skeletal density (8). This extrapolated variable can be expected to be less related to bone strength than areal BMD, since it does not take into account the important size component that influences the mechanical resistance.

Therefore, in terms of overall bone strength prediction, the areal BMD/BMC value is more informative than the isolated measurement of the volumetric trabecular density, since the former variable includes both the bone thickness and its integrated volumetric density. This statement does not mean that other variables which are more difficult to precisely assess, such as the microarchitecture of the trabecular network and/or the intrinsic “quality” of the mineralized tissue, do not play a contributing role in the resistance to mechanical force. Furthermore, it is obvious that a full understanding of the fundamental mechanisms that underlie the marked interindividual variability observed in bone mass gain will require separate analysis of how bone size, cortical thickness and volumetric trabecular density evolve during growth and to identify which are the main respective genetic and environmental factors that determine the development of each of these three important contributors to bone strength in adulthood.

Bone Mass Development

Before puberty, no substantial gender difference in bone mass of both the axial and appendicular skeleton has been reported (1). There is no evidence for a gender difference

in bone mass at birth. Likewise, the volumetric bone mineral density appears to be also similar between female and male newborns (9). This absence of substantial sex difference in bone mass is maintained until the onset of pubertal maturation (9–11). During puberty the gender difference in bone mass becomes expressed. This difference appears to be mainly due to a more prolonged bone maturation period in males than in females, with a larger increase in bone size and cortical thickness (10). Puberty affects much more the bone size than the volumetric mineral density (1,2,10). There is no significant sex difference in the volumetric trabecular density at the end of pubertal maturation (12–17). During puberty, the accumulation rate in areal BMD at both the lumbar spine and femoral neck levels increases four- to sixfold over a 3- and 4-yr period in females and males, respectively (18). The change in bone mass accumulation rate is less marked in long bone diaphysis (18). There is an asynchrony between the gain in standing height and the growth of bone mineral mass during pubertal maturation (10,18,19). This phenomenon may be responsible for the occurrence of a transient fragility that may contribute to the higher incidence of fracture known to occur when the dissociation between the rate of statural growth and mineral mass accrual is maximal (20,21).

Time of Peak Bone Mass Attainment

In adolescent females bone mass gain declined rapidly after menarche (18) to become not statistically significant 2 yr later (18). In adolescent males, the gain in BMD/BMC which was particularly high from 13–17 yr markedly declined thereafter, although it remained significant between 17–20 yr in both L2-L4 BMD/BMC and midfemoral shaft BMD (18). In contrast, no significant increase was observed for femoral neck BMD. In subjects having reached pubertal stage P5 and growing less than 1 cm/yr, a significant bone mass gain was still present in male but not in female individuals. This suggests the existence of an important sex difference in the magnitude and/or duration of the so-called “consolidation” phenomenon that contributes to the peak bone mass value.

Observations made with QCT technology also indicate that the maximal volumetric bone mineral density of the lumbar vertebral body will be achieved soon after menarche since no difference was observed between the mean values of 16- and 30-yr old subjects (22). This is in keeping with numerous observations indicating that bone mass does not significantly increase from the third to the fifth decade (13,15,16,23–36). Nevertheless, a few studies suggest that bone mass acquisition could still be substantial during the third and fourth decades. In any case, the balance of the collected data does not sustain the concept that bone mass at any skeletal site, in both genders, in all races and in any geographical area around the world continues to substantially accumulate until the fourth decade. In other words, that peak bone mass is reached in the mid-thirties does not seem to be a constant phenomenon of human physiology.

Note that the external frame of the bones can become larger during the adult life. This phenomenon has been documented by measuring the external diameter of several bones by radiogrametry (37–40). It may be the consequence of an increased endosteal bone resorption with enlargement in the internal diameter. This would result in a transient reduction in cortical thickness, leading to an augmentation in mechanical load on the remaining bony tissue. As a response to this increased load the number and/or activity of osteogenic cells on the periosteal side of the cortex would be enhanced, giving rise to compensatory growth with enlargement of the external diameter. Thus, such a modeling phenomenon would be a response to bone loss, tending to compensate the reduction in the mechanical resistance (41).

Peak Bone Mass Variance

At the beginning of the third decade, there is a large variability in the normal values of areal BMD in axial and appendicular skeleton (1). This large variance, which is observed at sites particularly susceptible to osteoporotic fractures such as lumbar spine and femoral neck, is barely reduced after correction for standing height, and does not appear to substantially increase during adult life (42). The height-independent broad variance in bone mass which is already present before puberty appears to increase further during pubertal maturation at sites such as lumbar spine and femoral neck (10,18). Note that in young healthy adults the biological variance in lumbar spine BMC is 4–5 times larger than that of standing height. It is also important to add that the variance in standing height does not increase during puberty (19).

CALCIUM-PHOSPHATE METABOLISM DURING GROWTH

Several physiological functions influence bone accumulation during growth. Animal studies have identified physiological mechanisms that sustain increased bone mineral demand in relation to variations in growth velocity. In this context, two adaptive mechanisms affecting calcium-phosphate metabolism appear to be particularly important, namely the increase in the plasma concentration of 1,25-dihydroxyvitamin D₃ (calcitriol), and the stimulation of the renal tubular reabsorption of inorganic phosphate (Pi). The elevation in the production and plasma level of calcitriol enhances the capacity of the intestinal epithelium to absorb both calcium and Pi. The increase in the tubular reabsorption of Pi results in a rise in its extracellular concentration. Without these two concerted adaptive responses, growth and mineralization cannot be optimal. Note that the increase in the tubular Pi reabsorption is not mediated by a rise in the renal production or in the plasma level of calcitriol (43).

Analysis of cross-sectional studies suggests that these two adaptive mechanisms could be essential to cope with the increased bone mineral demand during the pubertal growth spurt. An increase in plasma calcitriol concentrations has been reported during pubertal maturation (44). Both the pattern of this response and its consequence for intestinal calcium absorptive capacity in relation to pubertal bone mass acquisition remain difficult to document, since it would require a time-integrated estimate of the controlling (calcitriol and intestinal calcium absorption) elements. A tight relationship exists between the tubular reabsorption of Pi, the plasma Pi level, and growth velocity in children (45). A rise in plasma Pi during puberty has been reported (46,47). Precise quantitation of the relationship between changes in the regulatory component of the tubular Pi reabsorption and plasma concentration of Pi, and bone mass gain during puberty remains to be done. However, similar to the calcitriol—intestinal calcium absorption regulatory pathway, a correct evaluation would require a time-integrated assessment of the changes in the tubular reabsorption and plasma concentration of Pi during the period of accelerated bone mass gain.

The mechanism underlying the parallel rise in calcitriol and the tubular reabsorption of Pi has been recently clarified. In fact, experimental studies indicate that one single factor, namely insulin-like growth factor-I (IGF-I), could be responsible for the stimulation of both calcitriol production and tubular Pi reabsorption (TmPi/GFR) in relation to the increased calcium and Pi demand associated with bone growth (10,48). In humans, the plasma level of IGF-I rises transiently during pubertal maturation, to reach a peak

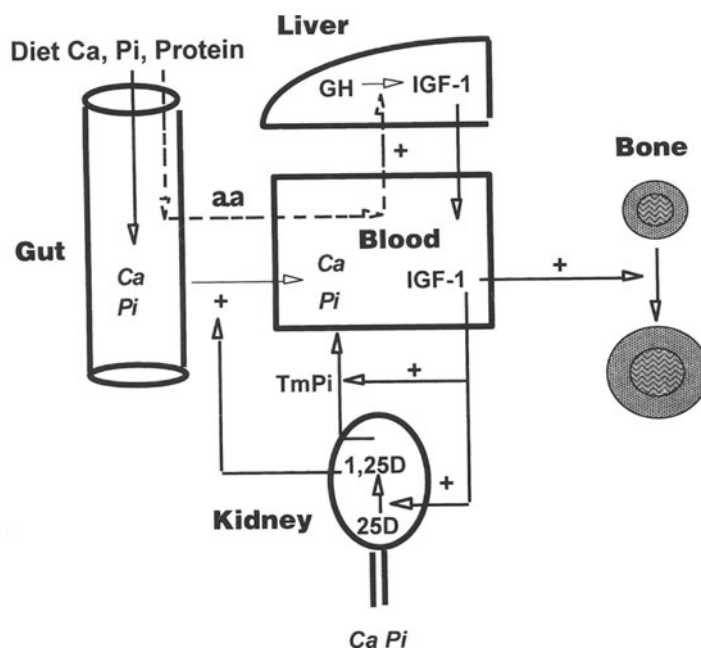


Fig. 1. Role of insulin-like growth factor-I (IGF-I) in calcium-phosphate metabolism during pubertal maturation in relation to essential nutrients for bone growth. During the pubertal bone growth spurt there is a rise in circulating IGF-I. The hepatic production of IGF-I is under the positive influence of growth hormone (GH) and essential amino acids (aa). IGF-I stimulates bone growth. At the kidney level, IGF-I increases both the 1,25-dihydroxyvitamin D (1,25 D) conversion from 25-hydroxyvitamin D (25D) and the maximal tubular reabsorption of Pi (TmPi). By this dual renal action IGF-I favors a positive calcium and phosphate balance as required by the increased bone mineral accrual. See text for references supporting this integrated physiological concept.

during mid-puberty, the maximal level, thus occurring at an earlier chronological age in females than in males (49). The role of IGF-I in calcium and phosphate metabolism during pubertal maturation in relation to essential nutrients for bone growth is illustrated in Fig. 1. The rise in the plasma levels of IGF-I, calcitriol, and Pi are correlated with the elevation in indices of the bone appositional rate such as alkaline phosphatase (50–53) and osteocalcin (52–55). Note that the plasma concentrations of gonadal sex hormones, as well as those of adrenal androgens (dehydroepiandrosterone and androstenedione), which increase before and during pubertal maturation, do not seem to accord with the accelerated bone mass gain (56–58). Whether differences in the adaptive responses which control calcium and phosphate homeostasis could play a role in the increased variance in lumbar spine or femoral neck BMD/BMC remain to be explored. As recently reviewed, the interaction between the growth hormone-IGF-I axis and sex steroids is quite complex (53). The effect of these interactions on the gains in bone size and mass during pubertal maturation, independent of their influence on the rate and duration of longitudinal growth, remains largely unknown.

Bone Biochemical Markers During Puberty

The interpretation of the changes in bone biochemical markers during growth is more complex than in adulthood, particularly for the markers of bone resorption (for review,

see ref. 53). The plasma concentrations of the bone formation markers are highest when the velocity of bone mineral accrual is maximal. This suggests that the two phenomena are related. The high urinary excretion of bone resorption markers, such as collagen pyridinium cross-links, observed during childhood, decreases after the growth spurt and reaches adult values at the end of pubertal maturation, i.e., at 15–16 and 17–18 yr of age in girls and boys, respectively (for review, *see ref. 53*). This probably reflects the decrease in the resorption rate associated with the reduction and arrest in longitudinal bone growth. In a longitudinal study in pubertal girls, bone turnover markers (osteocalcin, bone specific alkaline phosphatase, and collagen pyridinium cross-links) were modestly related to statural height gain, but they were not predictive of gains in either total bone mineral content or density as assessed by DXA (59).

DETERMINANTS OF BONE MASS GAIN

Many factors, more or less independently, are supposed to influence bone mass accumulation during growth. The list of these determinants classically includes heredity, sex, dietary components (calcium, proteins), endocrine factors (sex steroids, calcitriol, IGF-I), mechanical forces (physical activity, body weight), and exposure to other risk factors (16,60–64). Quantitatively, the most prominent determinant appears to be genetically related.

Genetic Determinant

As mentioned above the variability in BMD/BMC at the level of the lumbar spine and of the proximal femur, unrelated to changes in the statural height, increases during pubertal maturation. The contribution of heredity, compared to that of the environment, to this increased bone mass variability is not clearly elucidated. Genetic factors account for a large percentage of the population variability in BMD among age- and sex-matched normal individuals (60). Daughters of osteoporotic women have a low BMD (65). BMD is decreased among the relatives of 38 middle-aged men with severe idiopathic osteoporosis (66). To investigate the proportion of the BMD variance across the population explained by genetic factors, known as its heritability (67), two human models mainly have been used. In the twin model, within-pairs correlations for BMD are compared between monozygotic (MZ) twins, who by definition share 100% of their genes, and dizygotic (DZ) twins, who have 50% of their genes in common. Stronger correlation coefficients among adult MZ as compared to DZ twins are indicative of the genetic influence on peak bone mass, accounting for as much as 80% of lumbar spine and proximal femur BMD variance (60). Lean and fat mass are also genetically determined (68). Indeed, it appears that 80 and 65% of variance of lean and fat mass, respectively, are attributable to genetic factors. However, genetic factors affecting lean and fat mass have only little influence on lumbar spine or femoral neck BMD. These results differ from previous evidence of indirect genetic effects on bone mass occurring through the determination of lean body mass (69).

Parents–offspring comparisons have also shown significant relationships for BMD, albeit heritability estimates have been somewhat lower (in the range of 60%) than in the twin model (70). Actually, the magnitude of direct genetic effects on peak bone mass as evaluated in both human models may be overestimated by similarities in environmental covariates (62,71). We investigated correlations for bone mineral content, areal and

volumetric bone mineral density and bone area in the lumbar spine and femur (neck, trochanter, and diaphysis) in premenopausal women and in their prepubertal daughters (72). Regressions were adjusted for height, weight and calcium intake, to minimize the impact of indirect genetic effects as well as of dietary influences on bone mineral mass resemblance among relatives. The results indicate that despite great disparities in the maturity of the various constituents of bone mass before puberty with respect to peak adult values, heredity by maternal descent is detectable at all skeletal sites and affected virtually all bone mass constituents, including bone size and volumetric mineral density. Moreover, when daughters' bone values were reevaluated 2 yr later, while puberty had begun and bone mineral mass had considerably increased, measurements were highly correlated with prepubertal values and mother-daughter correlations had remained unchanged. Thus, a major proportion of this variance is due to genetic factors which are already expressed before puberty with subsequent tracking of bone mass constituents through the phase of rapid pubertal growth until peak bone mass is achieved. Interestingly, it appears that male to male and male to female inheritance of bone mass may differ substantially (71). It might be hazardous therefore to extrapolate genetic influences on bone mineral mass as identified in women to the male population, in which this question has virtually not yet been investigated.

In contrast to the clear heritability of peak bone mass, the proportion of the variance in bone turnover that depends on genetic factors, as assessed by various markers of bone formation and resorption, appears to be small (73). Hence, peak bone mass is very likely determined by numerous gene products implicated in both bone modeling and remodeling.

Among the multiple candidate genes harboring polymorphic loci so far investigated in relation to BMD and/or BMD changes, the vitamin D receptor (VDR)-3' end alleles are controversial (74–82). A meta-analysis combining 16 separate studies examined the relationship between VDR genotypes and BMD (82) and found that subjects with the BB genotype had a 2.4, 2.5, and 1.7% non-significantly lower BMD as compared to bb at the level of the femoral neck, lumbar spine, and distal radius, respectively. The more recently described association between VDR-5' start codon polymorphism (*FokI*) and BMD, at first observed in small cohorts of postmenopausal Mexican-American women (83), white premenopausal American women (84), and Japanese women (85), has not been confirmed in two larger European studies in healthy premenopausal women or prepubertal girls (86,87).

Several independent investigators have shown the importance of age, gene-environment, and gene-gene interactions to explain the inconsistent relationship between bone mineral mass and VDR-3' and 5'-genotypes. Thus, significant BMD differences between VDR-3' *BsmI* genotypes were detected in children (88,89), but were absent in premenopausal women from the same genetic background (89). Moreover, the latter study found that BMD gain in prepubertal girls was increased at several skeletal sites in Bb and BB subjects in response to calcium supplements whereas it remained apparently unaffected in bb girls, who had a trend for spontaneously higher BMD accumulation on their usual calcium diet (89,90). Accordingly, a model taking into account the early influence of VDR-3' polymorphisms, calcium intake and puberty on BMD gain has been proposed to explain the relation between these genotypes and peak bone mass (90). Interestingly also, several investigators have noted a significantly lower height among women and men with the VDR-3' BB compared to Bb or bb genotypes (89,91–94). Considering the relationship between body size and bone size, as well as the influence of calcium intake

on both body height and bone area during growth (94), it is tempting to speculate that VDR-3' alleles together with environmental calcium might exert an indirect and complex influence on peak bone mass by regulating skeletal growth. Altogether, these observations provide a possible physiological mechanism for the relationship between VDR gene polymorphisms and bone mass and emphasize the methodological limitations of earlier studies focusing on the association between VDR genotypes and BMD regardless of age and environmental factors. Moreover, other potential gene-environmental interactions, such as those involving physical exercise (95), as well as gene-gene interactions might further modulate the relationship between VDR gene polymorphisms and bone mass, as for instance, an interaction between VDR and estrogen receptor (ER) gene polymorphisms. In summary, VDR-3' and 5'-alleles are possibly weak determinants of bone mineral density, their effects being easily confounded by the influence of many other genes and environmental factors. Hence, VDR gene polymorphisms alone are not clinically useful genetic markers of peak bone mass, but could be one significant factor to explain some of the variability observed in the population.

Physical Activity

The responsiveness to either an increase or a decrease in mechanical strain is probably greater in growing than adult bones (96–98). Hence, the concept of public health programs aimed at increasing physical activity among healthy children and adolescents in order to maximize peak bone mass has been promoted. Several recent reports in children or adolescents involved in competitive sport or ballet dancing indicate that intense exercise is associated with an increase in bone mass accrual in weight-bearing skeletal sites (99–106). The question arises whether this increase in BMD/BMC resulting from intense exercise is translated into greater bone strength. A recent cross-sectional study in male elite-tennis players using peripheral QCT and side-to-side arm comparison indicates that the increase in BMC reflected an increased bone size which was associated with an augmentation in an index of bone strength. By contrast, no change in either cortical or trabecular vBMD was observed (107). Whether the same type of beneficial structural change for bone strength is observed at other skeletal sites, such as vertebral bodies and proximal femur, in response to different kinds of intense exercise during childhood and adolescence, remains to be documented. In terms of general public health, observations made in elite athletes cannot be the basis of recommendations for the general population, since intense exercise is beyond the reach of most individuals. Much more relevant is information on the effect of *moderate* exercise on bone mass acquisition. Some (63,108,109), but not all (110–114) cross-sectional studies have found a slightly positive association between physical activity and bone mass values in children and adolescents. However, the positive association found cross-sectionally was not confirmed by observational longitudinal studies relating bone mass gain to physical activity (112,115). Measurements of the duration, intensity, and type of physical activity that are based on recall are not very precise, particularly in children. Therefore, it is possible that negative findings could be ascribed to poor validity in the methods used to estimate physical activity. Recent controlled prospective studies carried out in prepubertal girls (116) or boys (117) indicate that exercise programs undertaken in schools, and considered on the average as *moderate*, can increase bone mass acquisition. These indicate that the growing skeleton is certainly sensitive to exercise, and suggest that prepuberty would be an opportune time for implementing physical education programs

consisting in various moderate weight-bearing exercises. Nevertheless, it remains uncertain to what extent the greater aBMD gain in response to moderate and readily accessible weight-bearing exercise is associated with a commensurate increase in bone strength (117). The magnitude of benefit in terms of bone strength will depend upon the nature of the structural change. An effect consisting primarily of an increased periosteal apposition and consecutive diameter will confer greater mechanical resistance than a response limited to the endosteal apposition rate leading essentially to a reduction in the endocortical diameter. There is a need for further studies aimed at examining the effects of mechanical loading components, such as magnitude and frequency of various types of exercise on the mass and geometry of bones in children and adolescents (118).

Studies in adult elite athletes strongly indicate that increased bone mass gains resulting from intense physical activity during childhood and adolescence are maintained after training attenuates or even completely ceases (102,104,119–121). Finally, the question whether the increased peak bone mass induced by physical exercise will be maintained into old age and confer a reduction in fracture rate remains open. A recent cross-sectional study of retired Australian elite soccer players suggests that this may not be the case (122). However, the lack of information on the peak bone mass values of these men does not allow one to draw firm conclusions about this observation.

Nutritional Factors

Puberty is considered to be a period with major behavioral changes and alterations in life-style. It is also assumed that important modifications in food habits occur during pubertal maturation, particularly in affluent societies. However, there is still a lack of quantitative and qualitative information regarding the evolution of both micro- and macronutrient intakes in relation to pubertal maturation. At the individual level, to what extent variations in the intakes of some nutrients in healthy, apparently well-nourished, children and adolescents can affect bone mass accumulation, particularly at sites susceptible to osteoporotic fractures, has received increasing attention over the last 10 yr. Most studies have focused on the intake of calcium. However, other nutrients such as proteins should also be considered.

CALCIUM

It is usually accepted that increasing the calcium intake during childhood and adolescence will be associated with a greater bone mass gain and thereby a higher peak bone mass (123,124). However, a survey of the literature on the relationship between dietary calcium and bone mass indicates that some (108,114,125–129), but not all studies (8,109,112,113,115,130) have found a positive correlation between these two variables. As with physical activity, several sets of cross-sectional and longitudinal data, including our own unpublished results on dietary calcium intake and bone mass accrual in female and male subjects ages 9–19 yr, are compatible with a “two threshold model.” On one side of the normal range one can conceive the existence of a “low” threshold, set at a total calcium intake of about 400–500 mg/d, below which a positive relationship can be found. Within this low range the positive effect of calcium would be explained merely by its role as a necessary substrate for bone mass accrual. On the other side of the normal range, there would be a “high” threshold, set at about 1600 mg/d, above which the calcium intake through another mechanism could exert a slightly positive influence on bone mass accrual. In addition, the levels of the two thresholds could vary according to

the stage of pubertal maturation. In our own cross-sectional study (10), a significant positive relationship between total calcium intake as determined by two 5-d diaries was found in females in the pubertal subgroup P1–P4, but not in the P5 subgroup. Furthermore, in the longitudinal study (18) when results were analyzed by taking into account the influence of age and pubertal maturation, the relationship between the absolute values of the calcium intake and the gain in BMD Z score suggested that calcium may be more important before than during pubertal maturation (130a).

Several intervention studies have been carried out in children and adolescents (94,131–135). Overall, these indicate greater bone mineral mass gain in children and adolescents receiving calcium supplementation over periods varying from 12 to 36 mo. The benefit of supplemental calcium has been greater in the appendicular than in the axial skeleton (94,132). Thus, in prepubertal children, calcium supplementation is more effective on cortical appendicular bone (radial and femoral diaphysis) than on axial trabecular rich bone (lumbar spine) or on the hip (femoral neck, trochanter) (94,132). The skeleton appears to be more responsive to calcium supplementation before the onset of pubertal maturation (132). As intuitively expected, this benefit may be particularly substantial in children with a relatively low calcium intake (94). In 8-yr old prepubertal girls with a spontaneously low calcium intake, increasing the calcium intake from about 700 to 1400 mg augmented the mean gain in aBMD of six skeletal sites by 58% as compared to the placebo group, after one year of supplementation (94). This difference corresponds to a gain of +0.24 standard deviation (SD). If sustained over a period of 4 yr such an increase in the calcium intake could augment mean aBMD by 1 SD. Thus, milk calcium supplementation could modify the bone growth trajectory and thereby increase peak bone mass. In this regard it is interesting to note that an intervention influencing calcium-phosphate metabolism and limited to the first year of life may also modify the trajectory of bone mass accrual. As a matter of fact, a 400 IU/d vitamin D-supplementation given to infants for an average of 1 yr was associated with a significant increase in aBMD measured at the age of 7–9 yr (136). The aBMD difference between the vitamin D-supplemented and non-supplemented group was particularly significant at the femoral neck, trochanter and radial metaphysis (136). These observations are compatible with the “programming” concept, according to which environmental stimuli during critical periods of early development can provoke long-lasting modifications in structure and function (137,138).

Another aspect to consider is that the type of the supplemented calcium salt could modulate the nature of the bone response. Thus, the response to administration of a calcium phosphate salt from milk extract appears to differ from those recorded with other calcium supplements. Indeed, the positive effect on aBMD was associated with an increase in the projected bone area at several sites of the skeleton and a slight increase in statural height (94).

This type of response was not observed when calcium was given as citrate maltate salts (132,133), carbonate alone (134), or carbonate combined with gluconate lactate (135). Interestingly, it was similar to the response to whole milk supplementation (139). But in this study (139), the positive effect on bone size could be ascribed to other nutrients contained in whole milk, whereas in the other study the tested calcium-enriched foods had the same energy, lipid, and protein contents as those given to the placebo-group (94).

It is important to consider whether or not the gain resulting from the intervention will be lost after discontinuation of the calcium supplementation. The answer to this question

remains uncertain. It could depend on the type of bone response observed, which could differ according to the type of the supplemented calcium salt. As mentioned above, with milk calcium phosphate salt (94), the increase in aBMD was associated with an increase of bone size. One year after discontinuing the intervention, differences in the gain in aBMD and in the size of some bones were still detectable, at the limit of statistical significance (94,140). Very recently, we observed that this difference was still present 3.5 yr after discontinuation of the supplementation (141). These results need additional confirmation by long-term follow-up of the cohort, ideally until peak bone mass has been attained, as well as by other prospective studies. Nevertheless, they apparently differ from results obtained with other calcium salt supplements (132,142). As a matter of fact, calcium given in other forms to pre- or peripubertal girls does not appear to modify bone size (132–135), nor to induce a persistent effect after stopping the intervention (143,144). This comparative analysis suggests that the positive effects observed on the aBMD or BMC gain with citrate maltate salts (132,133), or carbonate alone (134) could be primarily related to an increment in the volumetric density resulting from an inhibition of bone remodeling.

Despite a positive effect on mean aBMD gain there is still wide inter-individual variability in the response to calcium supplementation. As discussed above, it is possible that part of the variability in the bone gain response to calcium supplementation could be related to the VDR gene polymorphisms (89).

NUTRIENTS OTHER THAN CALCIUM

Among nutrients other than calcium, various experimental and clinical observations point to the existence of a relationship between the level of protein intake and either calcium phosphate metabolism or bone mass, or even osteoporotic fracture risk (145,146). Nevertheless, any long-term influence of dietary protein on bone mineral metabolism and skeletal mass so far has been difficult to identify. Apparently contradictory information suggests that either a deficient or an excessive protein supply could negatively affect the balance of calcium and the amount of bony tissue contained in the skeleton (145,146).

Despite these uncertainties, multiple animal and human studies indicate strongly that low protein intake *per se* could be particularly detrimental for both the acquisition of bone mass and the conservation of bone integrity with aging. During growth, undernutrition, including inadequate supply of energy and protein, can severely impair bone development. Studies in experimental animals indicate that isolated protein deficiency leads to reduced bone mass and strength without histomorphometric evidence of osteomalacia (145,146). Thus, inadequate supply of protein appears to play a central role in the pathogenesis of the delayed skeletal growth and reduced bone mass observed in undernourished children.

Low protein intake could be detrimental for skeletal integrity by lowering the production of IGF-I. Indeed, the hepatic production and plasma concentration of this growth factor, which exerts several positive effects on the skeleton, is under the influence of dietary protein (147). Protein restriction has been shown to reduce circulating IGF-I by inducing resistance to the hepatic action of growth hormone (148). In addition, protein restriction appears to decrease the anabolic actions of IGF-I on some target cells. In this regard, it is important to note that growing rats maintained on a low protein diet failed to restore growth when IGF-I was administered at doses sufficient to normalize its plasma concentrations.

Variations in the production of IGF-I could explain some of the changes in bone and calcium-phosphate metabolism that have been observed in relation to intake of dietary protein. Indeed, the plasma level of IGF-I is closely related to the growth rate of the organism. In humans, circulating IGF-I, of which the major source is the liver, rises progressively from 1 yr of age to reach peak values during puberty. As described above, this factor appears to play a key role in calcium-phosphate metabolism during growth by stimulating two kidney processes, Pi transport and the production of calcitriol (64,149). IGF-I is considered an essential factor for bone longitudinal growth, as it stimulates proliferation and differentiation of chondrocytes in the epiphyseal plate (150). It also plays a role on trabecular and cortical bone formation. In experimental animals, administration of IGF-I also positively affects bone mass (151), increasing the external diameter of long bones, probably by enhancing the process of periosteal apposition. Therefore, during adolescence a relative deficiency in IGF-I or a resistance to its action that could be due to an inadequate protein supply may result not only in a reduction in the skeletal longitudinal growth, but also in an impairment in widthwise or cross-sectional bone development.

In well-nourished children and adolescents, the question arises of whether variations in the protein intake within the “normal” range can influence skeletal growth and thereby modulate the genetic potential in peak bone mass attainment. There is a positive relationship between protein intake, as assessed by two 5-d dietary diary methods with weighing most food intakes (146,152), and bone mass gain during pubertal maturation (146). Since both bone mass and protein intake increase in both sexes during adolescence, it is not surprising to find a positive correlation between these two variables.

However, we found that the correlation remained statistically significant even after correcting for the influence of either age or pubertal stage. The association between bone mass gain and protein intake was observed in both sexes at the lumbar spine, the proximal femur, and the femoral mid-shaft. The association appeared to be particularly significant from pubertal stage P2–P4. However, these results should not be interpreted as evidence for a causal relationship between protein intake and bone mass gain. Indeed, it is quite possible that protein intake, which overall was related to the amount of ingested calories in our cohort, is to a large extent determined by growth requirements during childhood and adolescence.

As was the situation for other nutrients such as calcium, only prospective interventional studies will establish whether variations in protein intake within the range recorded in our western “well-nourished” population can affect bone mass accumulation during growth. Such prospective intervention studies should delineate the crucial years during which modifications in nutrition would be particularly effective for bone mass accumulation in children and in adolescents. This kind of information is of importance in order to make credible and well-targeted recommendations for osteoporosis prevention programs aimed at maximizing peak bone mass.

CONDITIONS IMPAIRING PEAK BONE MASS ATTAINMENT

Various genetic and acquired disorders can impair optimal bone mass acquisition during childhood and adolescence (153). In some endocrine disorders, such as Turner’s syndrome, Klinefelter’s syndrome, glucocorticoid excess, hyperthyroidism or growth hormone deficiency, low bone mass has been attributed to abnormalities in a single hormone. In diseases such as anorexia nervosa and exercise-associated amenorrhea, malnutrition, sex steroid deficiency and other factors combine to increase the risk of osteopenia or low

bone mass. This is probably also the case of various chronic diseases, which in addition may require therapies that can affect bone metabolism. Impaired bone growth has been frequently observed in chronic rheumatoid arthritis, chronic renal failure, cystic fibrosis, inflammatory bowel diseases, malignant hemopathies, hemoglobinopathies such as thalassemia major. Some conditions will be discussed further in this chapter.

Delayed Puberty

Epidemiological studies have provided suggestive evidence that late menarche is a risk factor for osteoporosis through a negative effect on PBM. In a cohort of men with a history of delayed puberty osteopenia has been reported (154). Delayed puberty or adolescence has been defined as the absence of any sign of puberty in a subject who has attained the upper normal limit of chronological age for the onset of puberty (155). This means an absence of increase in testicular volume at 14 yr in a boy or an absence of any breast development in a girl at 13 yr of age. The causes of delayed adolescence have been classified into permanent and temporary disorders (155). The permanent ones can be due to either hypothalamo-pituitary or gonadal failure (155). Among the temporary disorders, some can be explained by the presence of chronic systemic diseases, nutritional disorders, psychological stress, intensive competitive training, or hormonal disturbances such as hyposecretion of thyroid hormones or growth hormone, or hypercortisolism (155). However, the most common cause of delayed adolescence is the so-called “constitutional delay of growth and puberty” (CDGP). It is a transient disorder with, in some cases, a familial history of late menarcheal age of the mother or sisters, or a delayed growth spurt in the father. This condition has been considered so far as an extreme form of the physiological variation of the timing of the onset of puberty for which the “normal” range is about 8–12 and 9–13 yr of age in girls and boys, respectively. The onset of puberty is a complex process involving the activation of the hypothalamic-pituitary-gonadal axis and other endocrine systems such as the growth hormone-IGF axis of which the targets include factors influencing the bone mineral balance and the growth rate of the skeleton. Several mechanisms whereby CDGP may lead to a low peak bone mass have been suggested (156).

Anorexia Nervosa

Significant deficits in trabecular and cortical bone, which may result in osteoporotic fractures, have been observed in young adult women with chronic anorexia nervosa. Several factors can contribute to the reduced bone mass acquisition, including low protein intake resulting in a reduction in IGF-I production and thereby decreasing bone formation; low calcium intake enhancing bone resorption; estrogen deficiency; and glucocorticoid excess which interrupts normal acquisition of bone mineral and may contribute to increased bone loss (153).

Exercise-Associated Amenorrhea

Impaired bone mass acquisition can occur when hypogonadism and low body mass accompany intensive physical activity. As in anorexia nervosa, both nutritional and hormonal factors probably contribute to this impairment. Intake of energy, protein and calcium may be inadequate as athletes go on diets to maintain an idealized physique for their sport. Intensive training during childhood may contribute to a later onset and completion of puberty. Hypogonadism, as expressed by the occurrence of oligomenorrhea or amenorrhea, can lead to bone loss in females who begin training intensively after

menarche (153). Oligo-amenorrhea in long-distance runners was found to be associated with a decrease in BMD affecting more the lumbar spine than the proximal and midshaft femur (157).

CONCLUSIONS AND PERSPECTIVE

Peak bone mass is an important determinant of osteoporotic fracture risk. Hence, the interest of exploring ways of increasing peak bone mass in the primary prevention of osteoporosis. Bone mineral mass accumulation from infancy to postpuberty is a complex process implicating the interrelated actions of genetic, endocrine, mechanical and nutritional factors. It can now be better evaluated with the availability of noninvasive techniques able to precisely measure areal (a) or volumetric (v) bone mineral density (BMD) at various skeletal sites by DXA or QCT. From birth to PBM, which is attained in axial and in proximal femur by the end of the second decade, the increase in mass and strength is essentially due to an increment in bone size, vBMD changing very little during growth. Therefore, clinically the best simple estimate of bone strength is aBMD rather than vBMD which does not take into account the size of the bone. It can be estimated that in women an increase of PBM by 10%, i.e., by approx 1 standard deviation (SD) could decrease the risk of fragility fracture by 50%. Like standing height in any individual bone mineral mass during growth follows a trajectory corresponding to a given percentile or standard deviation from the mean. Nevertheless, this trajectory can be influenced by the environmental factors. On the negative side various chronic diseases and their treatment can shift downward this trajectory. On the positive side and most important in the context of primary prevention of adult osteoporosis, prospective randomized controlled trials strongly suggest that increasing the calcium intake or mechanical loading can shift upward the age-bone mass trajectory. Prepuberty appears to be an opportune time for obtaining a substantial benefit of increasing either the calcium intake or the physical activity. Further studies should demonstrate that the changes observed remain substantial by the end of the second decade and thus are translated into a greater peak bone mass. In this long term evaluation of the consequence of modifying the environment, it will be of critical importance to assess whether any change in densitometric and morphometric bone variables observed at PBM confers a greater resistance to mechanical strain.

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4

Peripheral Bone Mineral Assessment of the Axial Skeleton

Technical Aspects

Thomas F. Lang, PhD and Jeffrey Duryea, PhD

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INTRODUCTION

The purpose of the bone density measurement is to quantify the density or mass of bone mineral (calcium hydroxyapatite) in a medium consisting not only of the mineral itself, but also fat, muscle and bone marrow constituents as well as other biological materials. Measurements at central sites, such as the hip and spine, are inherently more complex than peripheral measurements because the bones are embedded in greater tissue thicknesses of more variable composition. Compared to peripheral sites (e.g., the calcaneus and forearm), where single-energy x-ray absorptiometry (SXA) and ultrasound measurements are available, central measurements require multiple-energy projection imaging techniques such as dual x-ray absorptiometry (DXA) or quantitative computed tomography (QCT).

The purpose of this chapter is to describe the underlying physical principles and principal error sources of DXA and QCT measurements as they apply to the central skeleton. In addition to describing the underlying principles and error sources, which have remained relatively constant since the inception of these techniques, this chapter

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will also provide information on the currently available whole-body DXA and QCT systems and some of the new approaches used by these systems to reduce cost and scan times and to improve precision and ease of use.

All x-ray based bone densitometry systems operate by comparing the x-ray attenuation of the tissue being measured to the attenuation of a reference system containing a mineral sample of known composition. In most systems, the mineral used in the reference sample is calcium hydroxyapatite ($\text{Ca}_{10}[\text{PO}_4]_6\text{OH}_2$). The comparison to a reference of fixed composition assumes that the composition of the mineral in the tissue does not vary significantly. Although this assumption is violated in a few disease conditions, it is generally not considered a problem for the clinical application of bone densitometry.

The term x-ray attenuation refers to the removal of x-ray photons from the incident beam of x-rays impinging on the tissue. At the x-ray energies most often used in bone mineral density measurements, the two common mechanisms are the photoelectric effect, in which an atom absorbs the incident photon, and Compton scattering, in which the photon is deflected by a collision with an atomic electron and loses an amount of energy which is a function of both the incident energy and the deflection angle. Photoelectric absorption and Compton scattering depend on the energy of the incident photon, the density of electrons in the tissue and the mean atomic number of the atoms in the tissue. Photoelectric absorption depends particularly strongly on the atomic number (Z) of the tissue and is more important in bone than in soft tissue because the bone contains a larger proportion of the higher- Z elements such as calcium ($z = 20$) and phosphorus ($Z = 15$). The fractional attenuation of incident x-ray photons as a function distance L in centimeters of tissue traversed is:

$$I/I_0 = \exp[-(\mu \cdot L)] \quad [\text{Eq. 1}]$$

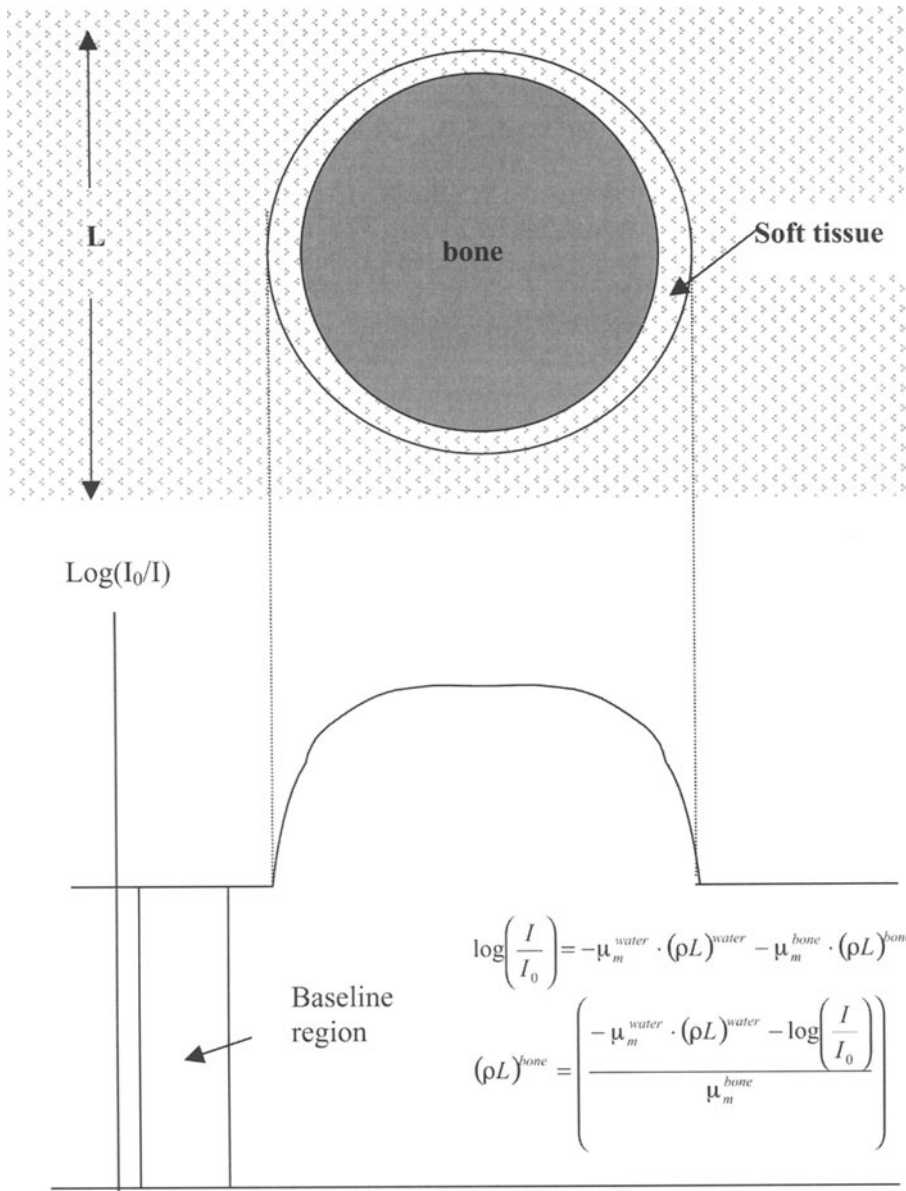
where I is the measured x-ray intensity exiting the tissue, I_0 is the incident x-ray intensity and μ is called the linear attenuation coefficient and is typically given in units of cm^{-1} . It is also possible to write this equation using the mass attenuation coefficient μ_m , which is typically given in cm^2/g , and which depends *only* on the photon energy and the elemental composition of the tissue:

$$I/I_0 = \exp[-(\mu \cdot \rho L)] \quad [\text{Eq. 2}]$$

ρ is the density in g/cm^3 of the tissue. As we will see later, if the μ_m of the tissue is known, it is possible to measure the areal density, ρL (g/cm^2), which is the primary measurement provided by DXA. ρL is the mineral mass per unit cross-sectional area measured at a given point in the two dimensional DXA image. On the other hand, QCT images are cross-sectional maps of the linear attenuation coefficient (μ), which are converted to maps of bone-equivalent density (typically mg calcium hydroxapatite per cm^3 of tissue) by comparison to a calibration standard scanned in the CT system.

PROJECTIONAL ABSORPTIOMETRY

In a DXA system, the areal density of bone mineral is measured at a specific location in the image based on the differential attenuations of two x-ray beams of different energies. Before discussing the procedure by which the areal density is calculated from the absorptiometric measurements, we will explore two simple examples, single-photon absorptiometry (SPA) (1–3) and dual-photon absorptiometry (DPA) (4–6). These two techniques are based on measurement of attenuation of mono-energetic radionuclide sources (DPA employed a ^{153}Gd radionuclide source with 44 and 100 keV photon



Profile of photon attenuation across idealized bone

Fig. 1. Idealized illustration of a SPA acquisition. The areal density of bone is calculated assuming a constant water-equivalent soft tissue baseline.

energies), and were employed prior to the introduction of DXA. However, the simple physics employed in SPA and DPA devices can be readily extended to understand the basic underlying principle of DXA, which utilizes a poly-energetic x-ray source.

Figure 1 shows a cross-section through an idealized limb consisting of an outer cylinder containing concentric rings consisting of subcutaneous fat, muscle and bone. A

intensity I_0 of photons of known energy E is emitted from the radionuclide source and impinges on the limb. On the other side, the output intensity I is measured. The fractional attenuation of the photon beam is given by:

$$I/I_0 = \exp \{ -[\mu_m^{\text{fat}} \cdot (\rho L)^{\text{fat}} + \mu_m^{\text{muscle}} \cdot (\rho L)^{\text{muscle}} + \mu_m^{\text{bone}} \cdot (\rho L)^{\text{bone}}] \} \quad [\text{Eq. 3}]$$

It is clear that to measure the areal density $(\rho L)^{\text{bone}}$, we must know the areal densities of the other components, assuming a priori knowledge of the mass attenuation coefficients for muscle, fat and bone at the photon energy E . In an SPA or in a single-energy x-ray system (SXA), this is accomplished by assuming a constant thickness of soft tissue of known composition around the bone of interest. Such an assumption may be realized experimentally by placing the limb of interest in a water bath to obtain a soft-tissue baseline, a known and constant attenuation of soft tissue. In this setting (Fig. 1), the composition of soft tissue is considered to be water equivalent, and of negligible thickness compared to the thickness of the water bath.

In projectional densitometry of central sites such as the spine or hip, it is not possible to use a single-energy approach in combination with a water bath because of high variability in the thickness and composition of the surrounding soft tissue. In this case, soft tissue attenuation is determined by measuring the differential attenuation of two photon energies (4–6). Figure 2 illustrates the principle of DPA by showing a cross-section through a segment of torso containing a lumbar vertebral body. The fractional attenuations of the two photon energies are given by two equations:

$$\begin{aligned} [I/I_0]_{\text{LE}} &= \exp \{ -[\mu_{\text{m,LE}}^{\text{ST}} \cdot (\rho L)^{\text{ST}} + \mu_{\text{m,LE}}^{\text{bone}} \cdot (\rho L)^{\text{bone}}] \} \\ [I/I_0]_{\text{HE}} &= \exp \{ -[\mu_{\text{m,HE}}^{\text{ST}} \cdot (\rho L)^{\text{ST}} + \mu_{\text{m,HE}}^{\text{bone}} \cdot (\rho L)^{\text{bone}}] \} \end{aligned} \quad [\text{Eq. 4}]$$

The fractional attenuation on the left hand side are measured experimentally, and the mass attenuation coefficients μ_m^{bone} and μ_m^{ST} are known a priori at the two energies. We can simplify this by calculating the log attenuation factors (LA), where $(\text{LA})_{\text{LE}} = \log [I/I_0]_{\text{LE}}$ and $(\text{LA})_{\text{HE}} = \log [I/I_0]_{\text{HE}}$.

$$\begin{aligned} (\text{LA})_{\text{LE}} &= -\mu_{\text{m,LE}}^{\text{ST}} \cdot (\rho L)^{\text{ST}} - \mu_{\text{m,LE}}^{\text{bone}} \cdot (\rho L)^{\text{bone}} \\ (\text{LA})_{\text{HE}} &= -\mu_{\text{m,HE}}^{\text{ST}} \cdot (\rho L)^{\text{ST}} - \mu_{\text{m,HE}}^{\text{bone}} \cdot (\rho L)^{\text{bone}} \end{aligned} \quad [\text{Eq. 5}]$$

Thus we have two equations in two unknowns, $(\rho L)^{\text{ST}}$ and $(\rho L)^{\text{bone}}$. We can solve for these two variables to obtain the areal densities of soft tissue and bone:

$$\begin{aligned} (\rho L)^{\text{bone}} &= \{ [(\text{LA})_{\text{HE}} - (\mu_{\text{m,HE}}^{\text{ST}} / \mu_{\text{m,LE}}^{\text{ST}}) \cdot (\text{LA})_{\text{LE}}] / [\mu_{\text{m,HE}}^{\text{bone}} - (\mu_{\text{m,HE}}^{\text{ST}} / \mu_{\text{m,LE}}^{\text{ST}}) \cdot \mu_{\text{m,LE}}^{\text{bone}}] \} \\ (\rho L)^{\text{ST}} &= \{ [-(\text{LA})_{\text{HE}} + (\mu_{\text{m,HE}}^{\text{bone}} / \mu_{\text{m,LE}}^{\text{bone}}) \cdot (\text{LA})_{\text{LE}}] / [-\mu_{\text{m,HE}}^{\text{ST}} + (\mu_{\text{m,HE}}^{\text{bone}} / \mu_{\text{m,LE}}^{\text{bone}}) \cdot \mu_{\text{m,LE}}^{\text{ST}}] \} \end{aligned} \quad [\text{Eq. 6}]$$

Thus, from measuring attenuation values at a given point using two photon energies, it is possible to measure the areal densities of the soft tissue and bone tissue components. However, this assumes that the mass attenuation coefficients for the bone and soft tissue components are known at the photon energy. While this may be readily measured or estimated for bone material, the composition of the soft tissue (proportion of lean and fat) is highly variable. This may be estimated by performing a log attenuation measurement in a region where there are no bone elements, and assuming that the relative proportions of lean and fat components in the soft tissue does not vary over the region being imaged. In this case (*see* Fig. 2), the ratio of the log attenuations for the two energies is calculated in this non-bone region.

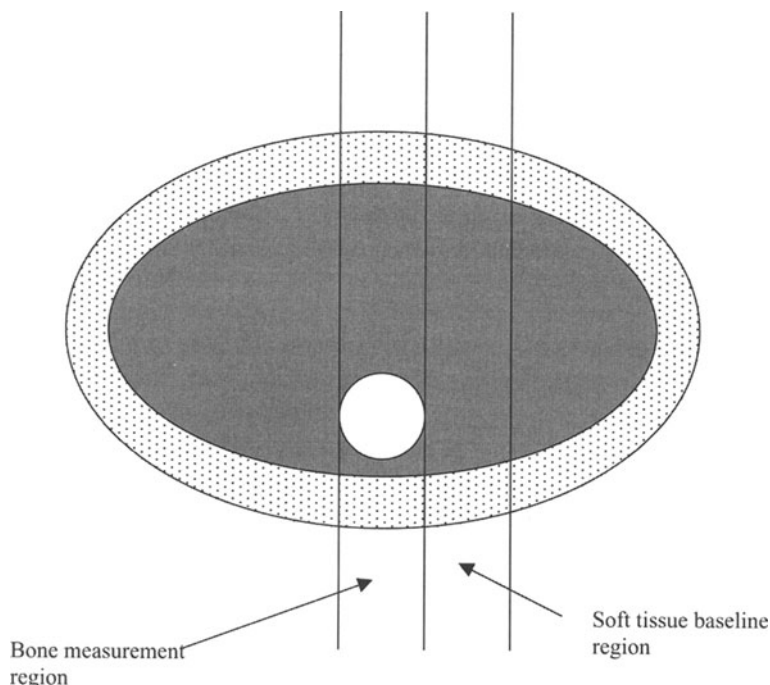


Fig. 2. DPA: Bone and soft tissue measurement regions.

$$R = (LA_{HE}/LA_{LE}) + (\mu_{m,HE}^{ST}/\mu_{m,LE}^{ST}) \quad [\text{Eq. 7}]$$

If R is now substituted for $(\mu_{m,HE}^{ST}/\mu_{m,LE}^{ST})$ into Eq. 6, it is possible to solve for the areal density of bone,

$$(\rho L)^{\text{bone}} = \{[(LA)_{HE} - R \cdot (LA)_{LE}]/(\mu_{m,HE}^{\text{bone}} - R \cdot \mu_{m,LE}^{\text{bone}})\} \quad [\text{Eq. 8}]$$

at any bone containing point in the dual-energy image.

DUAL X-RAY ABSORPTIOMETRY (DXA)

The previous section outlined the relatively simple principles whereby it is possible to measure the areal density of bone at a given point by measuring the attenuation of two known photon energies, and assuming a constant composition of soft tissue in the region being measured. DPA represented the first generation of dual-energy absorptiometric devices, but its wide application was hindered by a range of factors, including the need to replace and dispose of the radionuclide source. The limited photon flux produced by the radionuclide source also resulted in poor image quality and long acquisition times, and which limited measurement precision and ability to perform other procedures such as lateral spine measurements. DXA, which replaced the radionuclide with a x-ray source, was brought to market in the late 1980s (7–9). Since its inception, DXA has undergone continuing technical evolution, including the introduction of fanbeam devices (10–12) and the implementation of advanced detector technology to permit high-resolution morphometric imaging (11,13). Currently, there are several manufacturers of central DXA systems. Hologic (Waltham, MA) and GE-Lunar (Madison, WI) dominate the market. Central DXA systems are also offered by Norland (Fort Atkinson, WI) and

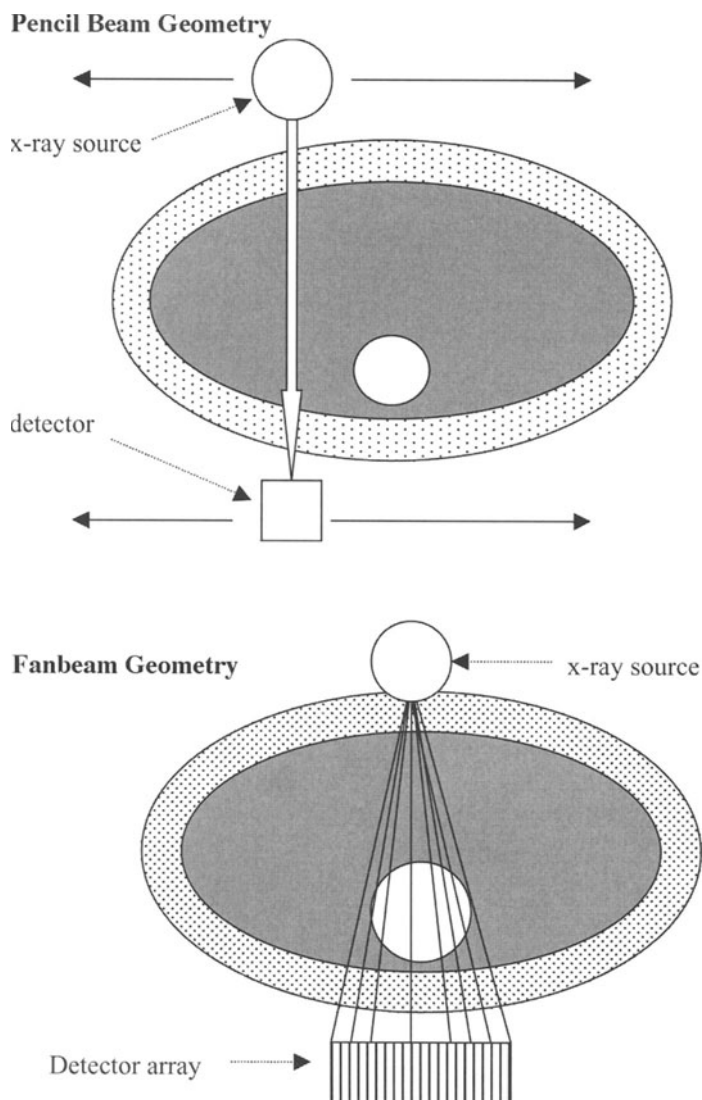


Fig. 3. DXA: Pencil and fanbeam geometry.

DMS (Montpellier, France). Systems range in cost from \$30,000 for the basic pencil beam systems to \$100,000 for a system equipped with fanbeam and C-arm option.

Figure 3 shows a schematic of a DXA system. An x-ray tube is mounted on a gantry, as is a detector system. Dual-energy spectra may be generated by placing a rare earth filter at the aperture of the tube, or by rapidly changing the kVp of the tube. The dual-peak energy spectrum provides an approximation of the dual-photon condition. The detector system measures the intensity I of x-rays transmitted through the tissue being imaged. An air calibration, typically performed daily or several times per day, provides a reference value I_0 , which is divided into I to provide the log attenuation value $\ln(I/I_0)$. Both the detector system and the x-ray tube are mounted on a gantry, which scans the tube

detector system across the object to generate the image. The gantry is responsible for mechanical support of the x-ray tube and detector, as well as for delivery and extraction of electronic signals. Attenuation measurements (LA_{HE} and LA_{LE}) from the detector system are conducted to a computer, where calibration factors are applied to generate images of areal density of bone and soft tissue. These images are then analyzed to calculate areal BMD for specific bones. In this section, we will discuss the components of the DXA system in detail.

System Geometries

There are two specific types of system geometries (Fig. 3), related to whether the DXA system contains a single detector, or an array of detectors. In the single detector scanning geometry, called the pencil beam geometry, the detector is positioned opposite from the x-ray tube, and these two components define a single ray path, or pencil beam, through the object being imaged. A projectional image of the bone of interest, or of the total body, is formed by scanning the pencil beam across the field of view in a rectilinear pattern. When a detector array is employed, a system geometry called a fanbeam is used. In this geometry, a fan-like set of ray paths is formed between the x-ray tube and the individual detectors. There were several motivations for the development of fanbeam systems. These included shorter imaging times (due to the ability to image the entire spine or hip in a single sweep with a wider detector), and clearer images based on the array of small (mm to sub-mm) detector elements. Both of these improvements potentially benefit precision due to reduced patient motion and improved definition of bone areas. Additionally, fanbeam systems have been promoted for vertebral fracture assessment based on lateral scans of the spine. When equipped with a rotating C-arm (Hologic QDR-4500 series), such systems can perform the imaging with the patient in the supine position, which improves patient comfort and presumably measurement quality.

X-Ray Tube

The x-ray tube produces x-ray photons having a wide range of energies. The maximum photon energy is equal to the peak kilovoltage (kVp) of the x-ray tube times one electron-volt. The peak energy of this broad distribution is called the effective energy. In order to approximate the dual-peak energy spectrum of ^{153}Gd , DXA manufacturers utilize two different methods to shape this relatively broad x-ray energy spectrum into narrower “high-energy” and “low-energy” peaks. GE-Lunar, Norland and Diagnostic Medical Systems filter the x-ray beam with a rare earth metal (9), which absorbs x-rays at the energy of its characteristic K-edge absorption line (Fig. 4). GE-Lunar DXA devices utilize cerium filters with a K-edge of 40.4 keV, resulting in a bi-modal x-ray energy spectrum with peaks at 38 and 70 keV. The Norland devices utilize Samarium filters with a K-edge of 47 keV, generating high- and low-energy peaks of 45 and 80 keV, respectively. Hologic DXA devices generate dual-energy x-ray spectra by switching the x-ray tube voltage on a rapid time scale (8). The Hologic QDR-4000 pencil-beam device generates a bi-modal x-ray energy spectrum by rapidly switching the x-ray tube voltage between 140 and 70 kV on a 8-ms time scale (Fig. 4). For the QDR-4500 fanbeam series, the kVp is switched between 100 and 140 kVp.

Detector Systems

The function of the detector is to determine the tissue attenuation at a given point in the patient by comparing the x-ray intensity measured at that point to the x-ray intensity

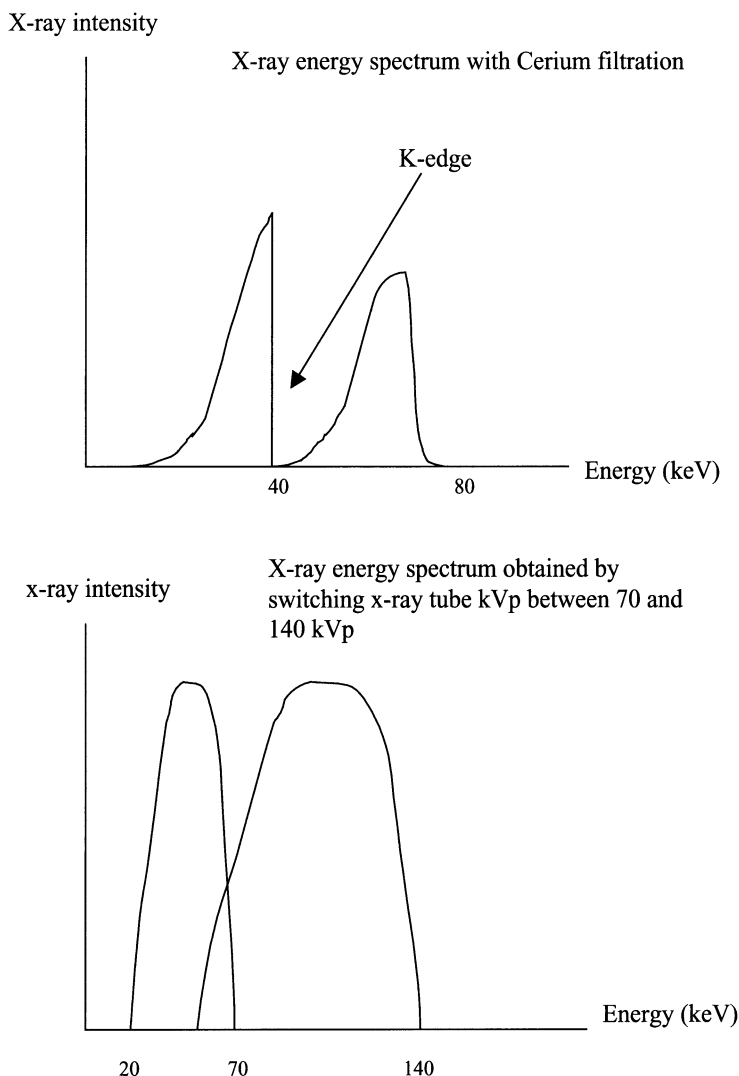


Fig. 4. Illustration of x-ray spectrum obtained with Cerium filtration (top) and kVp switching (bottom).

measured with no patient present. Pencil beam DXA systems typically utilize a single crystal of sodium iodide (NaI) mounted to a photomultiplier tube (PMT). The incident x-rays produce scintilla of visible light when they interact with the atoms of NaI crystal, and these scintilla are detected by the PMT, which produces an electrical current pulse which is a function of the energy of the x-ray. In the Lunar DPX series of DXA devices, the PMT signals are electronically processed to measure the energy and assign the x-ray count to a high- or low-energy x-ray window. Hologic DXA systems, on the other hand, rapidly switch the kVp to modulate the energy of the x-ray beam. This approach does not require that the detector system measure the individual photon energies. In this case, the detector system is operated in current-integration mode, which means that the PMT outputs an electrical current which is a function of the detected x-ray intensity. Fanbeam DXA systems, such as the Hologic 4500 series, and the Lunar Expert and Prodigy, utilize arrays of detectors. In the Hologic 4500 densitometers, the detectors arrays consists of

scintillation detector elements coupled to a matched to an array of photodiodes which convert the light output of the detectors into an electrical current proportional to the x-ray intensity. The Lunar Prodigy utilizes a solid state semi-conductor (ZnCdTe) detector array in which the energy of the incident x-rays is directly converted into an electrical current. The detector is coupled to electronic circuitry which sorts the individual x-rays into low- and high-energy counting bins on the basis of their energies.

Calibration of X-Ray Measurement for Bone Density and Mass

DXA manufacturers use two principal techniques to calibrate their dual-energy measurements to bone mineral density. In all Hologic DXA systems, the x-ray beam is passed through a continuously rotating filter wheel containing segments of bone- and soft tissue-equivalent materials as well as an air segment (8,14). The BMD of the patient at a given point is determined by comparing the high- and low-energy attenuation signals measured with the bone (which has a known BMD calculated to a fixed reference standard) and tissue segments in position to those obtained with the air segment. Unlike the Hologic Systems, the Lunar DXA systems operate on a fixed kVp and a bimodal x-ray spectrum is obtained by filtering the x-ray beam with a cerium filter (9). The calibration to bone mineral mass is obtained by a calibration algorithm known as basis material decomposition (15,16), which assumes that the any tissue can be represented as a combination of a bone-like and soft-tissue-like material, e.g., acrylic and aluminum. The calibration procedure generally involves measuring the high and low energy attenuations over a series of aluminum and acrylic calibration blocks of known thickness. The experimentally-defined thicknesses of the two basis materials are fit to polynomial functions of the high and low log attenuation measurements and the patient count rate data are mapped to equivalent thicknesses of aluminum and acrylic using these calibration relationships. The equivalent thicknesses of aluminum and acrylic are mapped to bone mineral density using a special calibration block containing a bone mineral reference with precise bone mineral content. Fat content is measured by applying the basis material decomposition method to a soft tissue area near the bone, with the fat content then being subtracted from the soft tissue thickness estimate.

Determination of BMC and BMD Values

DXA images are acquired and transferred to a computer system for analysis. The computer system has the tasks of extracting the bone mineral information from the dual-energy images, storing the values in a database and generating a report of the results. The low and high-energy images are combined to generate a two dimensional map of areal BMD. The images are processed by an edge detection algorithm in order to determine the bone boundaries. A fat correction is applied to the bone pixels based on a soft-tissue baseline performed in the area adjacent to the bone. In the spine, the software calculates the bone pixels and the positions of the intervertebral spaces. Typically, four regions of interest corresponding to the L1–L4 vertebral bodies are delineated. Each individual region of interest is bounded by the edges of the spinal column and the intervertebral boundaries. The BMC is calculated by computing the mean areal BMD within the region of interest and then multiplying by the projected area. The data are reported as the BMD, BMC, and areas of the individual vertebral bodies, as well as the total value corresponding to the summed BMC and Area values for the total number of bodies scanned. A similar analysis is applied to delineate anatomic regions of interest in the hip. The spinal and proximal femoral regions of interest and their clinical application is presented in Chapter 5.

Radiation Dose

The radiation doses for DXA are quite small, and are generally equivalent to the radiation exposure level associated with a few days of background radiation (17–19). Effective doses of on the order of 1–2 mSv have been reported for pencil beam DXA of the spine and hip (20). The radiation dose from fanbeam measurements is higher by about a factor of 10, potentially ranging up to 62 mSv (21).

Sources of Error

PRECISION AND ACCURACY ERRORS

Literature reports on the accuracy error of DXA in studies of excised bone samples *in vitro* have ranged from 2–4% (22–27). The precision of DXA depends on both machine, patient and operator-dependent factors (9, 11, 23, 24, 28–32). Machine dependent factors include changes in the effective energy of the x-ray tube, which may affect the stability of the BMD calibration and instabilities in the detector and associated electronics. However, this source of precision error is generally quite small, and for well-maintained systems the long-term precision *in vitro* is typically on the order of 0.5%. In pencil beam systems, statistical uncertainties in the mean BMD in a region of interest are a source of random precision error in BMD measurements. This is more of a problem with the photon counting systems than for the current-integrating systems because photon counting requires a low x-ray beam intensity to operate properly. Precision error *in vivo* is dominated by patient positioning, since the projected area of the spine or hip is affected by the rotation of the bone. Other sources of precision error include inconsistencies in the bone edges as determined by computer algorithms, as well as user interaction in the placement of anatomic markers (e.g., vertebral markers). For PA spine measurements, published values for the short term *in vivo* precision vary between 0.7–1.5%. Reported precision errors for lateral spine measurements are higher and have been reported to range up to 3%. Precision values for AP hip measurements range from 0.8–1.8%, with the lowest precision errors found for the total femur region. Precision errors are typically higher in the elderly than in younger subjects, because reduced bone density results in poorer bone edge definition as well as difficulty in placement of intervertebral markers.

INHERENT ERROR IN MEASUREMENT OF AREAL BMD-BONE SIZE DEPENDENCE

The first limitation is that both DXA BMC and areal BMD measurements scale with bone volume (33,34). Of two bones with the same volumetric density, the larger bone will have a higher areal density. Thus, the bone densities of two populations having different skeletal sizes and shapes are not directly comparable. Several literature reports have examined the impact of this technical problem on comparisons of DXA BMD between ethnic groups (35,36) as well as measurement of BMD in children (37–40).

DEPENDENCE ON FAT DISTRIBUTION

The bone mineral calculation of DXA assumes that human tissue is a two-component system consisting of bone and a soft tissue component of uniform composition. DXA systems resolve this problem by acquiring a soft tissue baseline in a region adjacent to the bone of interest, and using Eqs. 7 and 8 to correct for the admixture of adipose tissue. This approach assumes uniformity in the proportion of adipose material in the soft tissue. However, this assumption may not be correct for all body shapes, and thus the fat content overlying the bone may be different from that estimated from the soft-tissue sample. If the

amount of fat in the beam path is underestimated, this may artificially lower the BMD value. If it is less, it may artificially increase the BMD value. This problem has been extensively examined in the literature (41–43). Several studies have utilized CT images to delineate the distribution of adipose tissue and employed this information to check the assumptions of DXA. Tothill found that inhomogeneities in the adipose distribution resulted in errors up to approx 5% in AP spinal BMD and larger errors for lateral imaging (41). Svendsen et al performed a similar study and found accuracy errors of 5% for the AP spine, 10% for the lateral spine, and 6% for the femoral neck and total femur regions (42).

VARIABLE MAGNIFICATION

Images acquired by a fanbeam system are magnified by a system- and patient-dependent magnification factor (11–13,44,45). For a DXA image, the size of this magnification factor is a function of the distance of the bone from the detector and of the source detector distance. Because the magnification has similar effects on both the projected area and BMC, its effect on the diagnostic value of areal BMD is not considered to be significant. However, the patient-dependent magnification factor should be taken into account for studies in which geometric variables such as femoral neck width or the hip axis length are measured from DXA images.

BEAM HARDENING

Beam hardening is a phenomenon which results in a dependence of the BMD measurement on the total thickness of tissue. Beam hardening results from the fact that the radiation source is not monoenergetic but is in fact a distribution of x-ray energies. As the poly-energetic x-ray beam traverses the body, the lower energy photons in the continuous spectrum are preferentially absorbed. Thus, for particularly large body sizes, the energies of the x-ray beam, and thus, the log attenuations, are different than those obtained during the calibration measurement, which may involve a much smaller overall thickness of tissue. While the dual-energy calibration is able to account for most of the range of fat and lean tissue thicknesses, the fact is that at the extreme end, the effective shape of the x-ray spectrum differs from that used across the calibration measurement. Reports have investigated the magnitude of this effect on BMD measurements in conditions appropriate to hip and spine studies (11,25,46–48). Blake et al. performed a phantom study with body thicknesses varied between 15–25 cm and found a maximum BMD deviation from the known value of 0.23 g/cm², with a RMS deviation on the order of 0.01 g/cm², which is approximately equal to the measurement precision (46).

PATIENT-RELATED SOURCES OF ERROR

Several error sources may falsely elevate AP spinal BMD values. These include aortic calcium deposits, osteophytic growth near the endplates, hypertrophy of the posterior elements, hemangiomas and vertebral wedge and crush fractures. Some of these error sources may be circumvented by performing spinal DXA measurements in the lateral projection. While lateral vertebral BMD measurements do show greater ability to discriminate vertebral fractures than AP measurements (49,50), they have limitations as well. These include higher precision errors, as well as potential superposition of the L1 and L4 vertebral bodies by the ribs and ilium, respectively (33).

X-RAY COMPUTED TOMOGRAPHY

Computed tomography (CT) is a three-dimensional x-ray absorptiometric measurement which provides the distribution of linear attenuation coefficient in a thin cross-

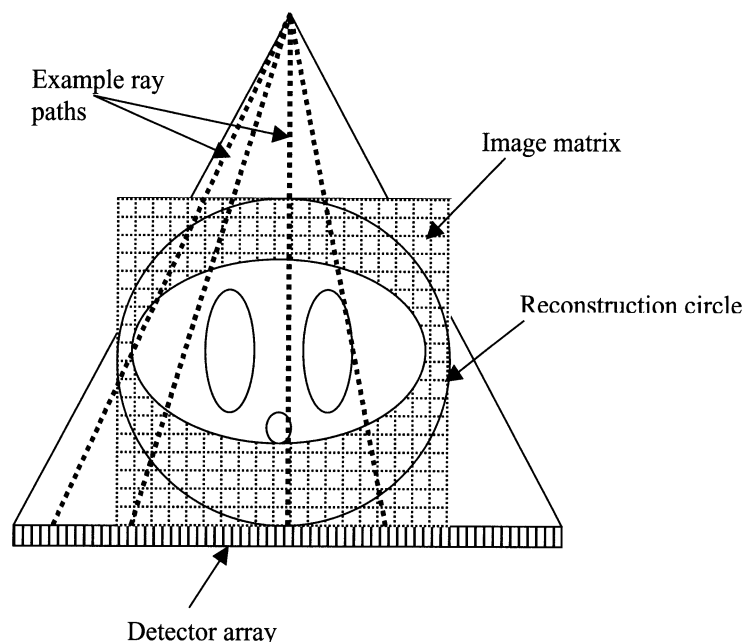


Fig. 5. Diagram illustrating geometry of a CT scan.

section of tissue. Figure 5 depicts the geometry of a CT measurement. The patient cross-section is contained within a fan of x-rays defined between the edges of the detector array and an x-ray point source. The x-ray attenuation of the patient is measured along ray-paths corresponding to the lines defined between individual detector elements and the x-ray source. Along the patient's length, the x-ray beam is shaped to radiate a relatively thin "slice" of tissue typically ranging from 1- to 10-mm. The fan of x-rays circumscribes a circular field of view, which is itself contained within a square image matrix, which typically consists of 512×512 square pixel elements, or "pixels." Because the image represents a slice of tissue, the picture elements have a thickness, and thus are volume elements, or "voxels." The dimensions of the voxels may be adjusted depending on the size of the organ being imaged. The voxel dimensions in the slice plane typically range from 0.9 to 0.2 mm, with slice thicknesses varying from 10 to 1 mm. The CT image is acquired when the x-ray source and detector rotate around the patient, and the absorption is continuously measured for each detector element. Through a 360° source-detector rotation, each voxel is intersected by several ray-paths. The x-ray absorption measurements taken at the different angles are recorded in a computer and combined in a process known as back-projection to calculate the linear attenuation coefficient at each voxel. Image data for multiple slices are acquired with motion of the patient table through the CT gantry. In older models of CT scanners, the patient table stepped in discrete increments, and a 360° rotation of the source/detector was performed at each position. In the newer model of helical CT scanners, the table and source/detector system move continuously, resulting in significant reductions in image acquisition time (51,52).

In the resulting CT image, the voxel values are based on the linear attenuation coefficients. Because these linear attenuation coefficients depend on the effective x-ray energy (which varies between CT scanner models and different kVp settings of the same

scanner), a simple scale, known as the Hounsfield scale, is used to standardize them. The gray-scale value of each voxel is represented as a Hounsfield Unit, given by:

$$HU_T = [(\mu_T - \mu_w)/\mu_w] \cdot 1000$$

where HU_T is the HU of a volume element of tissue and μ_T and μ_w are the linear attenuation coefficients of the tissue and of water, respectively. The HU scale is a linear scale in which air has a value of -1000 , water 0 , muscle 30 , with bone typically ranging from 300 to 3000 U.

The value of the Hounsfield unit for a given tissue type depends on several technical factors. First, if the sizes of the structures in the tissue are smaller than the dimensions of the voxel, the HU value is subject to partial volume averaging, in which the HU value is the average HU of the constituent tissues of the voxel, weighted by their volume fractions. For example, a $0.78 \times 0.78 \times 10$ mm voxel of trabecular bone is a mixture of bone, collagen, cellular marrow and fatty marrow, and HU is the volume-weighted average of these four constituents. Beam hardening is a second source of variation in HU. In a CT image, the result of this is that for the same tissue, attenuation coefficients at the outside of the patient are systematically higher than those in the interior. Although manufacturers of CT equipment have implemented beam-hardening corrections, the efficacy of these corrections varies between manufacturers and between technical settings on different machines.

QUANTITATIVE COMPUTED TOMOGRAPHY FOR SPINAL BONE MINERAL ASSESSMENT

Quantitative computed tomography (QCT) was developed in the late seventies as a method for measuring BMD in the metabolically-active trabecular bone in the vertebral bodies (53–56). In this approach, a lateral projection scan is utilized to localize the lumbar vertebral bodies and scans of 8–10 mm thickness are acquired through 2–4 contiguous lumbar vertebral levels (Fig. 6). The patient is imaged simultaneously with a bone mineral calibration standard, which is used to convert the native HU scale of the CT image to units of bone mineral density. The CT image is then processed using a software program which analyzes the calibration phantom to convert HU to BMD and then places a region of interest in the trabecular bone of the vertebral body (56–58). The program then calculates the mean BMD of the vertebral region of interest. This is either presented as an average of 2–4 vertebral levels. The radiation dose for this procedure has been reported as $80 \mu\text{Sv}$, a value which includes the dose of the lateral localizer scan (19).

PHYSICAL SIGNIFICANCE OF QCT MEASUREMENTS

CT BMD assessment is based on quantitative analysis of the HU in volumes of bone tissue. Typically, the BMD is quantified using a bone mineral reference phantom which is scanned simultaneously with the patient. In order to minimize the impact of beam hardening, the calibration phantom is placed as close as possible to the vertebrae, and is normally located under the lumbar spine of the patient. The calibration standard originally developed by Cann et al. at UCSF, and which is currently marketed by Mindways (South San Francisco, CA) consists of an acrylic wedge containing cylinders of solutions with varying concentrations of dipotassium hydrogen phosphate in water (54). An additional cylinder contained alcohol as a reference material for fat. A solid calcium hydroxyapatite-based calibration standard was later developed by Image Analysis



Fig. 6. Lateral scoutview. L1–L4 vertebrae are labeled. Bottom left: Axial slice through L3 vertebral body. Bottom right: Trabecular region of interest in L3 vertebral body.

(Columbia, KY) and by Siemens Medical Systems (Erlangen, Germany). The Image Analysis standard consists of rods with varying concentrations (200 mg/cm^3 , 100 mg/cm^3 , and 50 mg/cm^3) of calcium hydroxyapatite mixed in a water-equivalent solid resin matrix (59). During the analysis of the QCT image, regions of interest are placed (Fig. 6) in each of the calibration objects, and linear regression analysis is used to determine a relationship between the mean HU measured in each region and the known concentrations of bone-equivalent material. This calibration relationship is then used to convert the mean HU in the patient region of interest (for example, vertebra or proximal femur) into a concentration (reported in mg/cm^3 , i.e., the mass of bone per unit tissue volume)

of bone equivalent material in the region of interest. Unlike areal bone mineral density, the QCT density measurement is independent of bone size, and thus is more robust measure for comparisons of bone density between populations and potentially for growing children as well.

The major source of error in the QCT bone measurement is the phenomenon of partial volume averaging. Because the voxel dimensions in QCT measurements (0.8–1.0 mm in the imaging plane, 3–10 mm slice thicknesses) are larger than the dimensions and spacing of trabeculae, a QCT voxel includes both bone and marrow constituents. Thus a QCT measurement is the mass of bone in a volume containing bone, red marrow and marrow fat. A single-energy QCT measurement is capable of determining the mass of bone in a volume consisting of two components (e.g., bone and red marrow), but not in a three-component system (60,61). Resolving the mass fractions of bone, red marrow and marrow fat in the QCT voxel requires a dual-energy QCT measurement (62). Because fat has a HU value of –200, compared to 30 HU for red marrow and 300–3000 HU for bone, the presence of fat in the QCT volume reduces the depresses the HU measurement. Thus, the presence of marrow fat causes single-energy QCT to underestimate the mass of bone per unit tissue volume, an error which can be corrected using dual-energy acquisitions. The effect of marrow fat on QCT measurements is larger at the spine than at the hip or peripheral skeletal sites. Whereas the conversion from red to fatty marrow tends to finish by the mid-20s in the hip and peripheral skeleton, the vertebrae show a gradual age-related increase in the proportion of fat in the bone marrow which starts in youth and continues through old age (63). The inclusion of fatty marrow in the vertebral BMD measurement results in accuracy errors ranging from 5–15% depending on the age group. However, because the increase in marrow fat is age-related, single energy CT data can be corrected using age-related reference databases, and the residual error is not considered to be clinically relevant. Provided that the QCT scan is acquired at low effective energies (i.e., 80–90 kVp) (62), the population SD in marrow fat accounts for roughly 5 mg/cm³ of the 25–30 mg/cm³ population SD in spinal trabecular BMD. This residual error is not considered large enough to merit clinical use of dual-energy techniques, which are more accurate, but which have larger radiation doses and precision errors (61).

Precision errors ranging from 1–2% have been reported for spinal QCT (57,58). This precision error is attributable to several sources including the reproducibility of slice and region of interest positioning as well as scanner instabilities (64). Simultaneous calibration corrects to some extent for scanner instabilities, as well as for variable beam hardening depending on patient size and shape. Using a simultaneous calibration technique, the long-term CV of a well-maintained CT scanner should be close to 1%. The effect of variable patient positioning can be minimized by careful review of lateral localizer scans to ensure consistent slice placement and gantry angulations. Computer programs that place the region of interest automatically or semi-automatically may also be used to reduce precision errors.

COMMERCIALLY-AVAILABLE EQUIPMENT FOR SPINAL QCT MEASUREMENTS

QCT equipment typically includes a bone mineral reference phantom and software to process the CT images and report the results. All manufacturers of CT equipment offer a QCT option which may be purchased during installation or upgrade of the CT scanner.

Alternatively, a QCT package may be purchased from one of the manufacturers of specialized QCT equipment. The price for a QCT package can range between \$5,000–35,000, depending on whether the package only includes a calibration phantom or whether it also includes an analysis computer and software. The following vendors offer QCT systems:

IMAGE ANALYSIS (Columbia KY) offers a package which include a bone mineral calibration reference standard incorporating 200 mg/cm³, 100 mg/cm³, and 50 mg/cm³ concentrations of calcium hydroxyapatite in a water-equivalent resin matrix, an anthropometric torso phantom for longitudinal scanner QC and computer software to analyze spinal CT images, report BMD results and analyze longitudinal QC scans. The BMD measurement is based on acquisition of 10-mm thick slices through the T12-L3 vertebral bodies. The analysis software automatically places regions of interest in the calibration phantom and an elliptical region of interest in the anterior vertebral body. Other features of the Image Analysis software include a reporting module which calculates a T-score and which compares the patient data with an age-related normative curve. This QCT package may be purchased directly from the manufacturer or as an add-on in installation and upgrade of selected CT scanner models.

MINDWAYS (San Francisco, CA) offers a package which include a bone mineral calibration reference standard incorporating four solutions of different concentrations of dipotassium hydrogen phosphate as well as a sample of fat-equivalent material encased in a Lexan framework. Mindways also provides an anthropometric torso phantom for longitudinal scanner QC and computer software modules to analyze spinal CT images, report BMD results and analyze longitudinal QC scans. There are two different spinal BMD software modules. A two-dimensional analysis module measurement is based on acquisition of 10-mm thick slices through 2–4 contiguous vertebral bodies. The analysis software automatically places regions of interest in the calibration phantom and the user manually places an elliptical region of interest in the anterior vertebral body. A three-dimensional spine module is based on volumetric CT scans of the L1 and L2 vertebral bodies. The user interactively corrects the volumetric scans for rotation of the vertebrae in the AP and lateral planes, which allows the software to reconstruct a corrected 10-mm slice through the mid-vertebral body. The user then interactively places an elliptical region of interest in the anterior vertebral body. As with the Image Analysis software, other features of the Mindways software include a reporting module which calculates a T-score and which compares the patient data with an age-related normative curve. This QCT package may be purchased directly from the manufacturer.

CIRS (Norfolk, VA) offers a non-simultaneous calibration reference which consists of vertebra-shaped calcium hydroxyapatite inserts inside a simulated human torso. The user scans and analyzes images of the phantom to derive a scanner dependent, but not patient-specific relationship between concentration of calcium hydroxyapatite and HU. The analysis software applies this relationship to the mean HU inside a vertebral ellipse placed interactively by the user. The package includes reporting software which allows comparison of the patient BMD to a normative curve.

MEASUREMENT OF BMD USING VOLUMETRIC CT IMAGES OF THE SPINE AND HIP

QCT measurements based on single slice acquisitions are primarily applicable to relatively simple skeletal structures such as the spine and distal radius, but not to more

complex structures such as the hip, where minor variations in slice positioning can result in large variations in BMD. Moreover, single-slice scans are highly dependent on user interaction, particularly with respect to slice positioning. Three-dimensional CT scanning and image analysis procedures have become feasible with the advent of fast helical CT scanners and the cost reduction in computer processing power, which allows for inexpensive processing of the large volumes of CT data acquired in helical scans. Procedures have been developed for three-dimensional scanning of the spine and hip. In typical spine and hip protocols, the L1/L2 vertebrae and proximal femora (from superior aspect of femoral head to inferior aspect of lesser trochanter) are scanned with contiguous 3-mm slices. Using a radiation dose calculation developed by Kalender et al. (65), the radiation doses for these procedures have been estimated at 350 μSv and 1200 μSv for spine and hip protocols respectively.

One of the most powerful applications of helical CT scanning and three-dimensional image analysis is bone mineral assessment of the hip (66,67). Lang et al. have published a computer algorithm which processes three-dimensional CT images of the proximal femur to measure bone mineral density in the femoral neck, the total femur, and in a region which combines the trochanteric and intertrochanteric sub-regions of the Hologic DXA systems (Fig. 7) (66). Within each anatomic sub-region, the density, mass and volume are computed for the cortical and trabecular components as well as for the integral bone envelope. For trabecular BMD measurements, the precision of this method in vivo was found to range from 0.6 to 1.1% depending on the volume of interest assessed. In addition to the bone density measurements, the program can also measure aspects of the cross-sectional hip geometry. These measures include the cross-sectional areas and moments of inertia of the femoral neck, as well as the three-dimensional hip and femoral neck axis lengths. There is some evidence that the compartmental measurements provided by QCT of the hip may be of clinical and research interest. A range of studies have shown that trabecular bone may be important in the etiology of trochanteric as opposed to femoral neck fractures (68–70). In a study in vitro examining the effect of bone mineral density and geometry on proximal femoral fracture load, Lang et al. found that volumetric trochanteric trabecular BMD was highly correlated with fracture load when the femora were loading condition simulating a fall to the side. On the other hand, volumetric bone mineral density was only moderately correlated ($r^2 = 0.45$) with failure load in femora loaded in a single-legged stance condition. However, femoral neck BMD, cross-sectional area and axis length together explained 93% of the variation in failure load (66). The ability to examine both trabecular and cortical bone is an advantage for QCT of the hip, particularly in the case of therapies such as parathyroid hormone (PTH). In a QCT study of patients undergoing PTH therapy, Cann et al. found that cortical bone maintained its density but significantly increased its mass and volume (71).

The QCT manufacturer Mindways offers a software module which allows bone mineral assessment of the hip using helical CT scanning. In this procedure, called CTXA, a helical CT scan of the hip is reformatted into the AP projection and analyzed similarly to a DXA scan. The program allows the user to compute areal and volumetric integral BMD as well as volumetric trabecular BMD in regions comparable to those of DXA. The reporting module compares the CT-derived areal integral BMD measures to the NHANES III normative DXA data.

In the spine, the use of volumetric QCT measurements impacts precision more than discriminatory capability. Their ability to improve the precision of spinal measurements

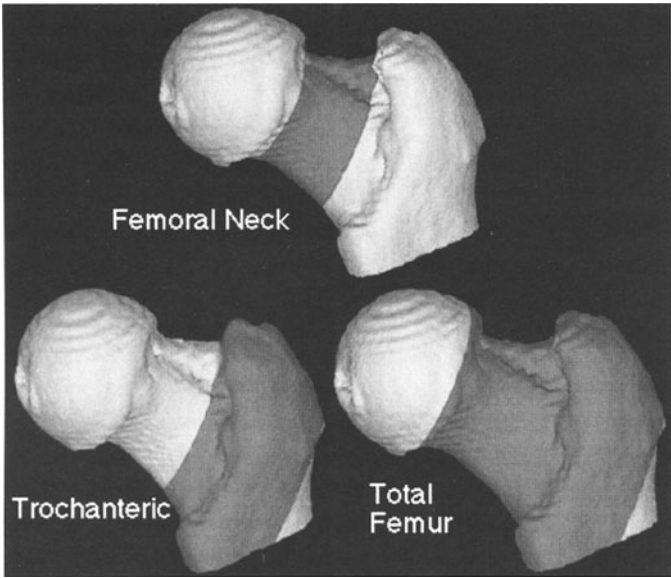


Fig. 7. QCT femoral regions of interest.

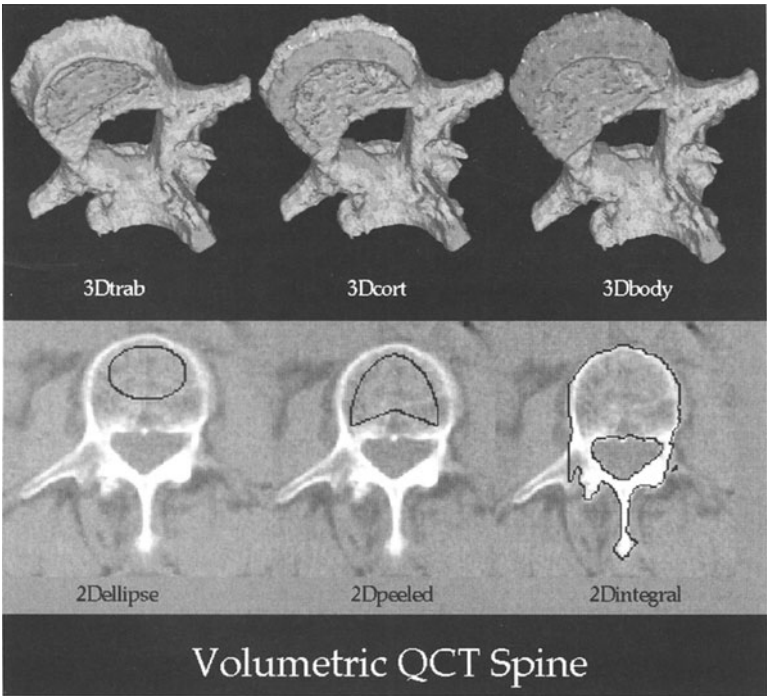


Fig. 8. QCT vertebral regions of interest. Top row of images are three-dimensional regions and bottom row are standard regions defined on single-slice scans.

relates to the use of three-dimensional anatomic landmarks to guide the placement of volumes of interest and to correct for differences in patient positioning which affect single slice scans. Currently, single-slice QCT techniques are highly operator dependent, requiring careful slice positioning and angulation as well as careful region of

interest placement. Lang et al. developed a volumetric spinal QCT approach in which an image of the entire vertebral body is acquired and anatomic landmarks such as the vertebral endplates and the spinous process are used to fix the 3D orientation of the vertebral body, allowing for definition of new trabecular and integral regions which contain most of the bone in the vertebral centrum, as shown in Fig. 8 (58). Although measuring a larger volume of tissue may enhance precision, these new regions are highly correlated with the mid-vertebral sub-regions assessed with standard QCT techniques and may not contain significant new information about vertebral strength. Consequently, volumetric studies of regional BMD, which examine specific sub-regions of the centrum that may vary in their contribution to vertebral strength (72–74), and studies of the cortical shell (75), the condition of which may be important for vertebral strength in osteoporotic individuals, are of interest for future investigation.

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5

Use of Bone Densitometry in the Clinical Management of Osteoporosis

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INTRODUCTION

Osteoporosis is sometimes termed the “silent epidemic” because early osteoporosis is asymptomatic, and significant bone loss may become evident only after a hip or vertebral fracture has occurred. Fractures, especially of the spine, hip, and wrist, are the clinical complications of osteoporosis. Initially, spine fractures tend to be asymptomatic but they are associated with significant morbidity as the severity and number of fractures increase. The most serious fractures are those of the hip, which contribute substantially to morbidity, mortality, and health care costs. Within a year of a hip fracture the mortality rate is as high as 20% with reduced functional capacity in 50% of surviving patients (1). Even the presence of clinical risk factors such as lifestyle, diet and family history of osteoporosis are relatively insensitive in predicting the presence of osteopenia (2). The pathophysiology of osteoporosis is multifactorial and complex. Fractures, the clinical manifestations of osteoporosis, depend on a variety of factors including the propensity to fall, visual acuity, response to falling, and bone strength (3,4). However, bone mass is the most important determinant of bone strength and accounts for up to 80% of its variance (5,6). Thus reduced bone mass should be a useful predictor of increased fracture risk (7). In fact, many prospective studies of older subjects have shown that levels of bone density at the spine or hip that are one standard deviation below the population mean increases the risk by a factor of two to three (8–11). Methods of measuring bone mineral density are pertinent to the assessment

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of osteopenia, identification of those individuals at risk of fracture, and identification of candidates for prevention or treatment strategies.

Conventional x-rays are readily available and fairly inexpensive. However, estimation of bone mineral content from the appearance of conventional radiographs is insensitive and inaccurate, since the subjective assessment is influenced by radiographic exposure factors, patient size, and film processing techniques (12,13). Bone mineral density must be reduced by as much as 40% before it can be detected reliably on radiographs (14). These factors have supported the need for objective, non-invasive methods of bone density measurements. These methods should be accurate, precise (reproducible), sensitive, inexpensive and involve minimal exposure to ionizing radiation.

In the last 25 yr, considerable effort has been expended on the development of non-invasive methods for assessing bone status in the axial and peripheral skeleton. Current techniques include radiographic absorptiometry (RA), single x-ray absorptiometry (SXA), dual x-ray absorptiometry (DXA), quantitative computed tomography (QCT) and quantitative ultrasound (QUS) (15–20). These techniques vary in precision, accuracy, and discrimination, and differ substantially in methodology, in clinical and research utility, and in general availability. Other chapters describe the fundamentals and use of ultrasound measures and the technical basis for radiographic absorptiometries. This chapter discusses the clinical utility of radiographic densitometry in evaluating bone status and provides a survey of its clinical use in the evaluation of osteoporosis.

PRESENTATION OF BMD

Units of Measure

Bone fragility can be assessed by measuring bone mass in selected regions of interest. Both areal bone density and volumetric bone density are commonly referred to as bone mineral density (BMD). Since bone mass, (measured in grams), is dependent on the size of the bone and the size of the region of interest, areal BMD measures are typically normalized to bone area and expressed as grams per unit area (mg/cm^2). Measures of volumetric bone density are expressed in grams per unit volume (mg/cm^3). The most common sites of measurement are areas where fragility fractures are likely to occur, (e.g., the spine, proximal femur, and the distal forearm). All these regions can be measured using dual x-ray absorptiometry (DXA), (areal density) or quantitative computed tomography (volumetric density). In this chapter, we make no distinction between areal or volumetric density except where noted. For anterior-posterior (AP) spine and Total Femur BMD assessments by dual energy x-ray absorptiometry, the units of measure have been standardized across manufacturers such that the BMD measured on different devices and different manufacturers is on average the same.

Reference Population

To be clinically useful, BMD results for individual patients are usually related to values obtained from a healthy reference population. There are no universal standards for these reference data. It is ideal to use, when available, a population that is most closely related to the patient in terms of sex, race, and country of origin. Reference populations are usually described in terms of the mean BMD and standard deviation of the population as a function of age, sex, and race, and in some cases nationality. Figure 1 shows the sex and race mean BMD values for the Total Femur ROI from the National Health and Nutrition

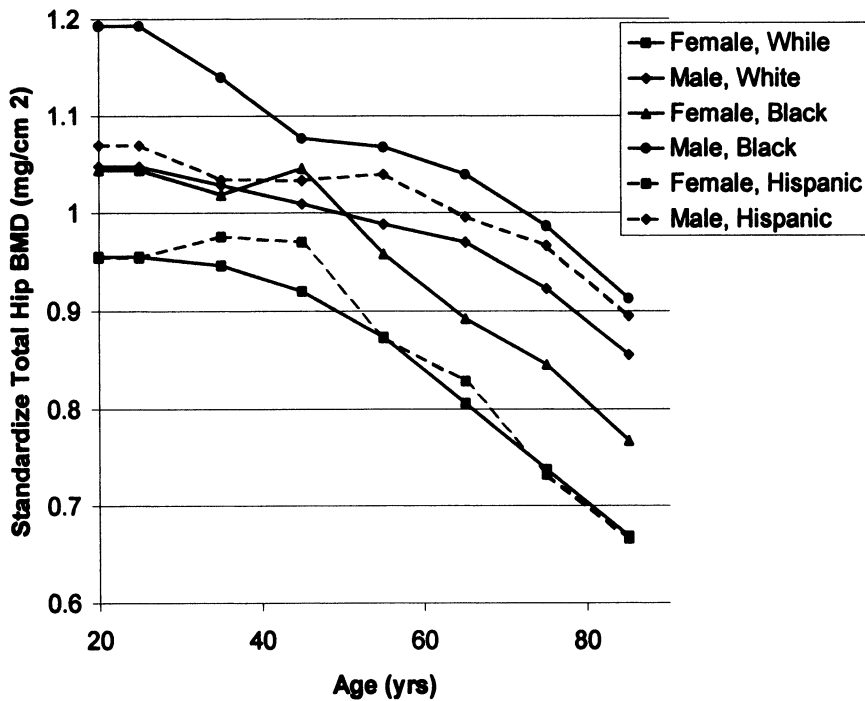


Fig. 1. Reference data from the NHANES III Study (21). BMD is shown in standardized BMD units.

Examination Survey (NHANES) III (21). Bone density can vary by as much as 25% between Black males and Caucasian females of the same age. For many patients, adequate reference groups are not available. Therefore, the clinician must use available data and be aware of possible ethnic and geographical differences.

T-Scores

The T-Score is an expression of the comparison of a patient's BMD to young adult (or peak) reference values. It is expressed in units of the young adult population standard deviation, SD, or as a percent of the reference population's value. The $T\text{-Score}_{sd}$ represents the number of SDs a BMD measurement is above or below the mean bone mass of a young normal population matched for sex and race. In units of standard deviations, $T\text{-Score}_{sd}$ is defined as:

$$T\text{-Score}_{sd} = (BMD_{\text{patient}} - BMD_{\text{peak}}) / \text{Std.Dev}_{\text{peak}} \quad [\text{Eq. 1}]$$

where BMD_{patient} is the patient's BMD value, BMD_{peak} is the young adult reference BMD value, and $\text{Std.Dev}_{\text{peak}}$ is the population standard deviation at that peak value. As a percentage of the population's peak value, $T\text{-Scores}_{\%}$ is defined as:

$$T\text{-Score}_{\%} = (BMD_{\text{patient}} / BMD_{\text{peak}}) \cdot 100 \quad [\text{Eq. 2}]$$

Z-Scores

Z-Scores are comparisons of the patient's BMD to an age-matched reference population value and can also be represented in units of population standard deviations or as

a percent of the reference population's value. The $Z\text{-Score}_{sd}$ expresses the number of standard deviations a subject differs from the mean value for an age, sex, and race matched reference population. Z-scores are not used to define osteoporosis since their use would result in the apparent prevalence of disease not increasing with age. The Z-score, however, is quite useful clinically in assessing a patient's skeletal status relative to his or her peers. In units of standard deviation, $Z\text{-Score}_{sd}$ is defined as:

$$Z\text{-Score}_{sd} = (\text{BMD}_{\text{patient}} - \text{BMD}_{\text{age-matched}}) / \text{Std.Dev}_{\text{age-matched}} \quad [\text{Eq. 3}]$$

where $\text{BMD}_{\text{patient}}$ is the patient's BMD value, $\text{BMD}_{\text{age-matched}}$ is the reference population BMD value at the same age as the patient, and $\text{Std.Dev}_{\text{age-matched}}$ is the population standard deviation at the patient's age. As a percentage of the population's age-matched BMD value, $Z\text{-Scores}_{\%}$ is defined as:

$$Z\text{-Score}_{\%} = (\text{BMD}_{\text{patient}} / \text{BMD}_{\text{age-matched}}) \cdot 100 \quad [\text{Eq. 4}]$$

A sample calculation of the T- and Z-Score is shown graphically in Fig. 2. A 70-yr-old female patient has a BMD that is 2.4 population standard deviations below the mean BMD value of a young reference population and 1 population standard deviation below the mean BMD value for women her age in the reference population. Thus, the $Z\text{-Score}_{sd}$ is -1.0 while her $T\text{-Score}_{sd}$ is -2.4 .

INTERPRETATION OF BMD RESULTS

WHO Criteria

The World Health Organization (WHO) established a definition of osteoporosis based on bone density (Table 1). These recommendations, originally intended for population-based studies, have been widely used as diagnostic and treatment thresholds for individuals. This approach is certainly easy to apply. However, there are inherent problems to such a simplistic threshold approach. For instance, it is heavily dependent on estimates of young adult reference means and standard deviations, moreover, differing patterns of bone loss among skeletal measurement sites are not taken into consideration (22). Finally, it ignores the fact that there is a continuous increase in fracture risk with decreasing BMD rather than a stepwise increase.

NOF Recommendations

The National Osteoporosis Foundation (NOF) is a nonprofit health organization dedicated to promoting bone health and to reducing the prevalence of osteoporosis and fractures. It has made treatment recommendations based on BMD and other risk factors (23). For instance the NOF has recommended:

Initiate therapy to reduce fracture risk in women with BMD T-scores below -2 in the absence of risk factors and in women with T-scores below -1.5 if other risk factors are present.

These guidelines are an enhancement to the WHO criteria in that they consider risk factors that may affect fracture risk in addition to just the BMD T-Score. Despite these recommendations the precise BMD threshold values for the initiation of treatment are a matter of continued debate.

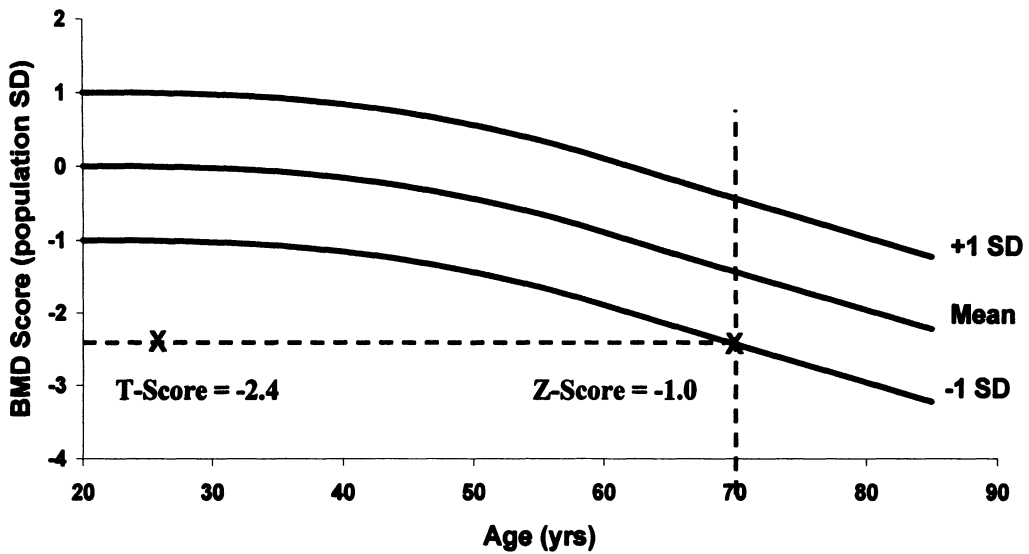


Fig. 2. Calculating T and Z Scores. Graph is for the Total Femur from the NHANES III study for female Caucasians. The patient shown is a 60-yr-old Caucasian woman with a BMD of 0.64 mg/cm². Her Z-score is -1.0 while her T-Score is -2.4.

Table 1
WHO Definitions of Osteoporosis on BMD or BMC Value^a

Classification	Description
Normal	A value of BMD greater than 1 SD below the average value of a young adult ($T > -1$)
Low bone mass (Osteopenia)	A value of BMD more than 1 SD below the young adult average but not more than 2.5 SD below ($-2.5 < T \leq -1$)
Osteoporosis	A value of BMD more than 2.5 SD below the young adult average value ($T \leq -2.5$)
Severe (established) osteoporosis	A value for BMD more than 2.5 SD below the young adult average and there has been one or more osteoporotic fractures

^aSee ref. 36.

Fracture Risk

The diagnosis of osteoporosis currently relies heavily on bone mass measurements, and in a clinical setting the essential role of bone densitometry is to identify patients at higher risk for fractures. There have been several large epidemiological studies to estimate fracture risk in light of BMD measures at several different anatomical sites. The Study of Fractures (SOF) assessed hip fracture risk in 8,134 Caucasian women over age 65 yr from BMD measurements of the proximal hip, lumbar spine, forearm, and the calcaneus (8). Figure 3 shows the relative fracture risk as a function of Z-Score using the SOF results. A postmenopausal Caucasian woman with a Z-Score of -2 at the total hip had a risk of hip fracture 5.4 times that of women with a Z-Score of 0 at the same age.

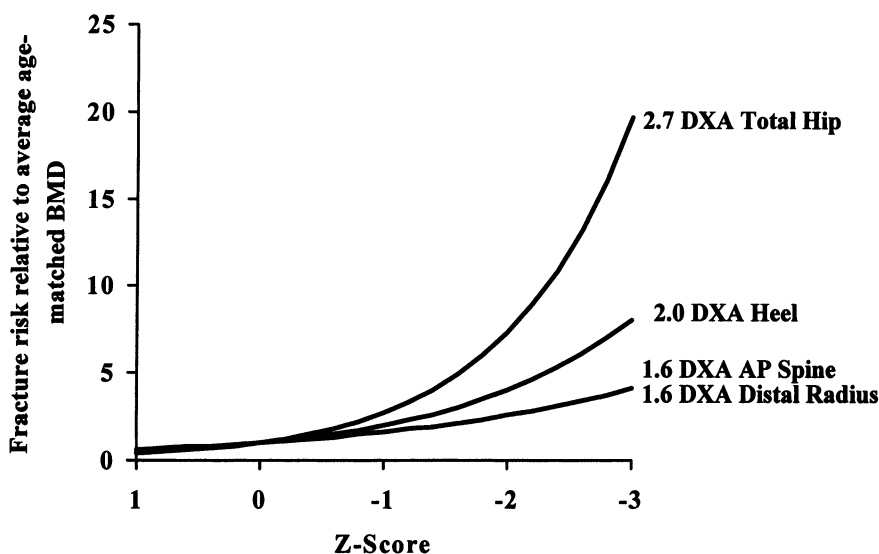


Fig. 3. Fracture risk associated with the BMD measurement from different measurement sites taken from the SOF study. The risk of fracture relative to women at the mean BMD is shown as a function of increasing Z-Score.

Ross and Wasnich et al. proposed the concept of remaining lifetime fracture probability (RLFP) (24). RLFP is a method of expressing absolute fracture probabilities calculated from long-term epidemiological studies. The model considers a variety of BMD measurement sites in light of ethnicity and gender. The clinician can choose specific treatment strategies for individual patients and view how the RLFP changes over the short term (the next year) and the long term (remaining life expectancy). Similarly, Melton et al. (25) introduced the concept of lifetime fracture risk. This model uses clinical risk factors to determine the risk of fracture in the patient's remaining lifetime.

APPLICATION OF BMD MEASUREMENTS

Who Should be Tested?

Bone densitometry is becoming widely used in routine medical practice but it should be used only in the context of the individual's clinical situation. Testing is generally not indicated unless the results could influence a management decision. Although still controversial, some recommendations have been developed to determine when a bone density scan is appropriate in the assessment of osteoporosis. For instance, the National Osteoporosis Foundation (23) recommends that BMD testing should be performed on:

- All postmenopausal women under age 65 who have one or more additional risk factors for osteoporotic fracture (besides menopause).
- All women aged 65 and older regardless of additional risk factors.
- Postmenopausal women who present with fractures (to confirm diagnosis and determine disease severity).
- Women who are considering therapy for osteoporosis, if BMD testing would facilitate the decision.
- Women who have been on hormone replacement therapy for prolonged periods.

However, the guidelines for reimbursement from insurance companies and government coverage in many cases drive the decision to perform the measurement. A 1998 Medicare law provides reimbursement for bone density tests in five groups including:

1. Estrogen-deficient women at clinical risk for osteoporosis.
2. Individuals (man or woman) with vertebral abnormalities indicative of osteoporosis, low-bone mass or vertebral fracture.
3. An individual (man or woman) receiving long-term glucocorticoid therapy.
4. An individual (man or woman) with primary hyperparathyroidism.
5. Individuals (men or women) being monitored to assess the efficacy of an FDA-approved osteoporosis drug therapy.

The guidelines state that these individuals will be covered for repeat BMD tests at a frequency of every two years. Patients receiving high glucocorticoid doses (i.e., ≥ 7.5 mg/d of prednisone) may qualify for 6-mo or yearly measurements, and individuals who are first assessed using a peripheral BMD site may require an axial (spine, hip) BMD measurement.

How to Use BMD Measurements

The best use of any bone densitometry technique depends on the nature of the clinical problem, the age of the patient, and technical factors (26). The primary purpose of measuring BMD should be to assess fracture risk in individual patients to make clinical decisions designed to minimize fracture risk. In fact, a recent meta-analysis demonstrated that BMD is a better predictor of the risk of fracture than cholesterol is of coronary disease or blood pressure is of stroke (9). Bone densitometry measures are appropriate if the result will influence subsequent clinical decisions. As an example, a postmenopausal woman with severe symptoms of estrogen deficiency would be appropriately treated with estrogen regardless of the bone density. However, if the density value will affect a decision to undertake hormone replacement therapy, then testing is clearly defensible. An international panel has recently outlined the assumptions implicit in the use of BMD measures (27). The main consensus statements are listed below.

- Bone mass measurements predict a patient's future risk of fracture.
- Osteoporosis can be diagnosed on the basis of bone mass measurements even in the absence of prevalent fractures.
- Bone mass measurements provide information that can affect the management of patients.
- The choice of the appropriate skeletal measurement site(s) may vary depending on the specific circumstances of the patient.
- The technique chosen for bone mass measurements should be based on an understanding of the strength and limitations of the different techniques.
- Bone mass information should be accompanied by a clinical interpretation.

Diagnostic Algorithms

Several examples of diagnostic algorithms have been proposed for the use of BMD measures. The NOF has suggested an approach based on widespread screening to enhance the detection of subjects with low BMD and high fracture risk. Those recommendations are based heavily on the assumption that the patient will consider a treatment option if BMD is found to be reduced (23). Using this approach, the measurement of BMD should not be considered unless the patient would consider the use of an intervention. An alternative approach that depends on the presence of clinical risk factors for osteoporosis

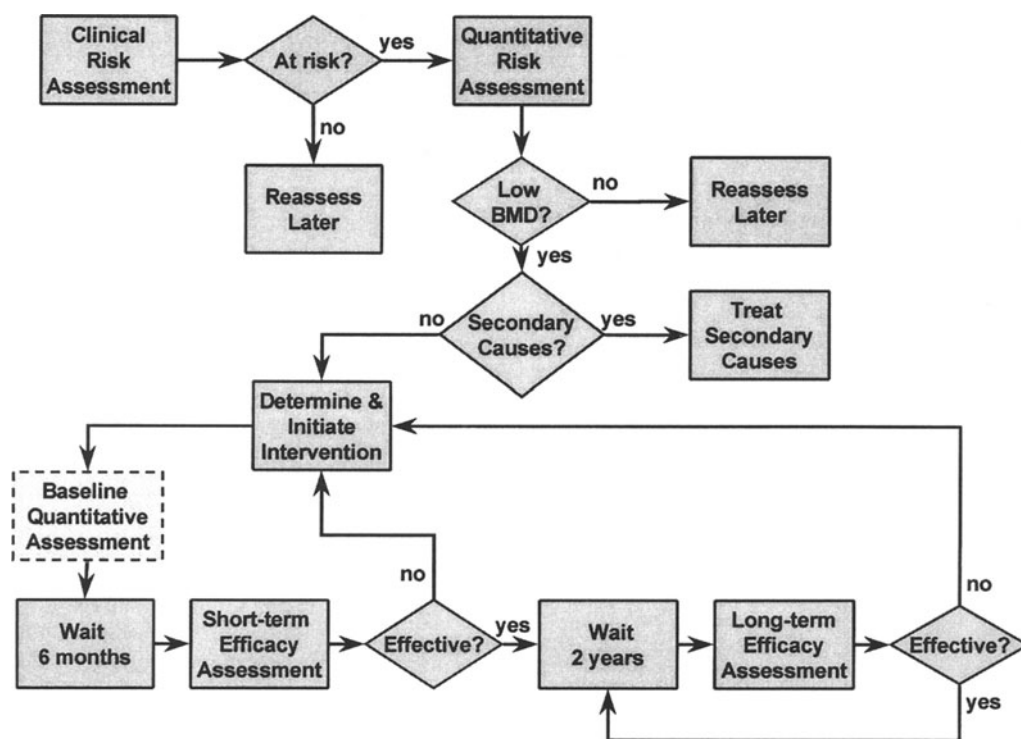


Fig. 4. Diagnostic algorithm for the diagnosis of osteoporosis.

to prompt a BMD measurement is shown in Fig. 4. The patient is initially assessed to determine if he/she has risk factors for low BMD. If not, then a reassessment is considered at a later date. If risk factors are present, a quantitative BMD measurement is performed. This may be any one of the following: BMD assessment of the hip and spine; peripheral BMD measures (forearm, heel, or hand); quantitative ultrasound assessment of the heel, forearm or hand; and x-ray examination of the spine to identify prevalent fractures. If the quantitative assessment does not show low BMD the patient is reassessed at a later date.

If the patient has low BMD the clinician should determine if the osteoporosis is due to secondary causes such as inflammatory disorders (RA), bone marrow disorders, defects of connective tissue synthesis (OI), malabsorption, endocrinological disorders (e.g., hyperparathyroidism, hypogonadism), or immobilization. Conversely, when a patient with a known metabolic bone disorder seeks medical attention, a BMD measurement may be obtained to determine the magnitude of the deleterious affect of the disorder on the skeleton. In this setting, the use of the Z-score is particularly helpful.

WHICH SITE TO MEASURE

Available Sites

Skeletal sites contain varying proportions of trabecular and cortical bone, that may differ in their rates of bone loss. AP measurements of the spine by DXA include approximately 66% trabecular bone, while measures of the femoral neck include about 75% cortical bone. The proximal forearm consists of predominantly cortical bone (95%),

while the distal and ultradistal measurement sites contain about 50% trabecular bone (28,29). However, osteoporosis is a systemic disease and loss of bone usually occurs at all sites (albeit at different rates) and low-bone density at a variety of measurement sites is associated with the risk of osteoporotic fracture (8,27). One could thus assume for diagnostic purposes that bone density could be measured at any site. However, measurements of the lumbar spine in the very elderly are commonly falsely increased by the presence of soft tissue mineralization related to degenerative arthritis (8,30).

In a meta-analysis, Marshall et al. (9) showed that the predictive value of available BMD fracture measures are similar when considering overall fracture risk. However, the risk of hip fracture is best predicted by BMD measures of the proximal femur (26).

Limitations

The clinician should be aware of the limitations of BMD measures, such as the effect of disease or medication on the site of measurement. For example, changes in BMD are often more marked in the trabecular bone of the spine and can be detected earlier than at the hip or wrist. In the elderly, where degenerative spinal disease may be prevalent, BMD assessed by spinal QCT or at an appendicular site (e.g., the femur) may be more effective for risk assessment. Alternatively, lateral DXA scanning of the lumbar spine reduces the influence of degenerative changes and may be a more suitable measurement approach in the elderly (31).

Combining Measurement Sites to Gain Diagnostic Power

The question of whether more information would be gained by making multiple measurements has been examined. Several studies have noted the discordance in BMD measurements between skeletal sites and have suggested that low BMD can be overlooked if BMD is measured at a single site (32). Wasnich and colleagues found that combining BMD measurements from multiple sites improved the prediction of incident vertebral fracture (32). Lu et al. showed that considering all the four measurement sites of the hip increased the relative fracture risk from 2.4 (only one of the four sites osteoporotic) to 8.6 (all four sites osteoporotic). Others have suggested that hip BMD measurements provide an excellent assessment of hip fracture risk but the addition of measurements at the spine, calcaneus or radius adds little new information (17,33). However, there are not adequate data available to support routinely considering the results from more than one measurement site. Further investigation is required to clarify the role of multiple measurements. Nevertheless, more than one site is frequently assessed when practical (e.g., hip and lumbar spine).

TREATMENT CONSIDERATIONS

Serial measurements are potentially useful to monitor the natural history of osteoporosis and to assess response to therapy. The choice of anatomical site for longitudinal testing depends on two variables, the rate of change of bone mass at that skeletal site and the precision of the measurement. In general, the observed change in bone density should be 2.8 times the long-term precision error for the technique in order for the change to be significant (34,35). When performed by an experienced technologist the precision of serial DXA scans of the lumbar spine is approx 1–2% and is approx 2–3% at the femoral neck. Thus changes in bone density of at least 3–6% at the spine and 6–8% at the femoral

neck are required to be considered significant. The rate of bone loss in the lumbar spine in early menopause is on average 2% per year when assessed by DXA, but may range from less than 1% by radial SXA to more than 5% per year by spinal QCT (36). Thus, the choice of interval between measures must be informed by knowledge of the precision of the technique and expected change at the site of measurement (37). Testing too infrequently can mean that patients who are rapidly losing bone are not detected; but testing too frequently is unlikely to yield useful information. For patients with secondary conditions producing osteoporosis, such as corticosteroid therapy, a rapid rate of bone loss can occur and more frequent measurements (every 6 mo) may be useful (38).

Repeated measurements also may be useful for assessing the response to therapy. In applying serial measures, however, one must be aware of the phenomenon of “regression to the mean,” which can confound interpretation of serial changes (39).

Poor compliance can impact the long-term preventive strategy of osteoporosis management. For example, poor compliance is often observed in patients treated with hormone replacement therapy (HRT). One study in the UK revealed that almost 40% of women prescribed HRT were not using the treatment after 8 mo (40). Compliance with treatment for osteoporosis may be enhanced through regular doctor/nurse follow-up, including education about the benefits of treatment and which side effects to expect. There is some evidence to suggest that knowledge of bone density enhances the acceptance of prescribed lifestyle changes and therapeutic intervention (41,42). Monitoring can also be used to follow patients after cessation of therapy (43).

Measurement Precision

Errors are inherent in quantitative measurements. There are two types of measurement errors: accuracy error and precision error (44). Accuracy error (also termed “validity”) refers to the ability of a technique to measure the true value. To evaluate accuracy errors the true values of the measured parameters must be known. For most BMD techniques, accuracy has generally been defined as the degree to which bone densitometry is able to estimate the mineral content. The measured BMC or BMD is compared directly to the ash or volumetric density of the measured bone. One should note that for clinical applications only the fraction of the accuracy error that varies from patient to patient in an unknown fashion is of relevance. The other fraction, i.e., the systematic, constant inaccuracy, can be averaged across subjects and is not important for two reasons. First, for diagnostic use of the technique the reference data will be affected by the same systematic error and thus the T- and Z-scores remain unaffected. The relative relationship between healthy and osteoporotic bone is maintained. Second, when monitoring change in bone density, the systematic error is present in both baseline and follow up measurement and does not contribute to the measured change (45). For these reasons, small systematic accuracy errors are of little clinical significance provided they remain constant.

Precision error (also termed reliability) reflects the reproducibility of a technique. It measures the ability of a technique to measure a parameter consistently over multiple measurements. In the clinical context of monitoring, precision is more important than accuracy. There are two major sources of precision error: equipment and technologist/patient. Precision errors can be further separated into short term and long-term precision errors.

SHORT-TERM PRECISION

Short-term precision errors characterize the reproducibility of a technique and are useful in describing the limitations of measuring changes in skeletal status. Generally

short-term precision errors are assessed from measurements performed either on the same day or extending over a period of several weeks. Precision can be expressed as absolute (standard deviation) or relative (percentage) variability.

Short-term precision is determined by calculating the mean and standard deviation of two or more repeated measurements on each subject. The individual means and SDs for all the subjects are then averaged. Nonetheless, since precision was measured for different subjects, there is the question of how to pool them. Glüer et al. (45) reported that the correct estimate is not given by the arithmetic mean of the individual subjects' precision errors, instead by RMS SD. However, it is a popular convention to express precision data as the coefficient of variation (CV-relative) by dividing the SD by the mean for all subjects and expressing the result as a percentage (45,47). In the special case where two measurements are made on each subjects the CV can be expressed as Eq. 5:

$$CV_{RMS} = \frac{\sqrt{\sum_{j=1}^m d_j^2/m}}{\sum_{j=1}^m \bar{x}_j/m} \cdot 100\% \quad [\text{Eq. 5}]$$

where d_j is the difference between the first and the second measurements for the duplicate measurements, m is the number of paired measurements and x is the mean of the paired measurements.

LONG-TERM PRECISION

Long-term precision estimates are used to evaluate instrument stability. The assessment of long-term in vivo precision is complex since the variability of the data may be due to imprecision of the technique as well as to true biological changes in the measurement (e.g., age-related bone loss). For repeated measurements taken on the same subject over time, the variability about the regression curve (i.e., the standard error of the estimate [SEE]) could be taken as an estimate of the long-term precision error. This is expressed as (34):

$$CV_L = \frac{\sqrt{\sum_{j=1}^m SEE_j^2/m}}{\sum_{j=1}^m \bar{x}_j/m} \quad [\text{Eq. 6}]$$

SEE results were predicted from the regression model.

Even when the underlying changes in BMD are truly linear, long-term precision errors would be expected to be larger than short-term precision due to small drifts in instrumental calibration, variation in patient positioning, and changes in soft tissue composition (34).

STANDARDIZED PRECISION

Precision expressed as a ratio of standard deviation and mean does not take into account the different biological range or responsiveness of the different sites (47). A technique with poor precision but with the ability to demonstrate larger changes (responsiveness) over time is preferable to a competing high precision and low responsiveness approach. Responsiveness depends on many factors including the measurement site and the technique. Different approaches have been suggested to standardize CV for easy comparison of different measurement techniques, although no consensus has been

reached (47). One approach is to divide the CV by the annual percentage change in the parameter due to age (responsiveness) or treatment as derived from normative data (48). The weakness of this approach is that the annual rate of change provided by the manufacturers is usually obtained on different populations and is cross-sectional. A true comparison of techniques could be derived if rates of change were obtained on the same population. On the other hand, Miller et al. (49) has suggested standardizing precision errors by calculating the ratio of percentage precision to percentage range of results (5th-95th percentile).

$$sCV = CV\% / (4SD / \text{Mean}_{\text{pop}}) \quad [\text{Eq. 7}]$$

where SD is the population standard deviation and mean is the average for all subjects. One criticism of this approach is that it depends on the spread of the subject group and so the results are difficult to compare with other reports. Glüer et al. (47) has suggested using

$$CV_s = CV \cdot [\text{response rate}(\text{reference technique})] / [\text{response rate}(\text{technique studies})]$$

where CV is the uncorrected precision error and sCV the standardized precision error of the technique that is being studied. Glüer (47) noted that for bone densitometry the ideal reference technique is the posterior–anterior DXA of the lumbar spine, since it has the lowest reported CV.

When are Two Measurements Significantly Different?

When assessing the suitability of a technique for serial measurements, the most important parameter is precision. This is because the least significant change (LSC) between two measurements on a single subject is related to the precision error. This can be expressed as:

$$LSC = z \cdot CV \sqrt{(1/n_1) + (1/n_2)} \quad [\text{Eq. 8}]$$

where z is the value for the desired level of confidence, CV is the precision (could be absolute or relative) and n_1 and n_2 are the number of measurement at each time point. It has been suggested that long-term precision should be used instead of short term. So, for 95% confidence ($z = 1.96$) and a single measurement at baseline and follow-up, the LSD is given by $1.96^2 \cdot CV$.

TREND ASSESSMENT MARGIN

The least significant change is highly dependent on the power (Z) set to detect the change. Glüer has proposed using the term trend assessment margin (TAM) for an 80% confidence level ($z = 1.26$) for two sided test or 90% confidence for a single-sided test. $TAM = 1.8 \cdot CV$.

MONITORING TIME INTERVAL

The monitoring interval is the time required to observe a significant change in the majority of the subjects. This is defined as:

$$MTI = LSC / \text{response rate} \quad [\text{Eq. 9}]$$

For example, for a technique with a precision error of 1% the LSC (95% confidence) will be 2.8%, if the bone loss is 3% per year, the MTI will be 1 yr. The concept of LSC

and MTI can be useful for the identification of fast losers of bone. The inverse of the MTI (i.e., ratio of the response rate to the precision error) has been termed the longitudinal sensitivity.

NONRESPONDING PATIENTS

In interpreting a treatment effect, clinicians should be aware of the statistical phenomenon known as regression to the mean (50). This phenomenon is due in part to measurement error and in part to biological variation. The clinical consequence is that a subject that has apparently experienced a particularly large gain in bone mineral in the first measurement interval is likely to show a reduced increase in BMD in the second interval (the subject's response has "regressed" toward the mean of the group). Similarly, a subject who has lost an unexpectedly large amount of bone in the first interval is likely to show less of a loss, even a gain, in the second interval. Over a longer period of time, the extremes will be less deviant from the mean because some of the errors of repeated measurements will smooth the results (51). Cummings et al. (39) demonstrated this effect using data from two randomized, double blinded, placebo-control trials of alendronate and raloxifene treatment. They concluded that effective treatment for osteoporosis should not be changed because of moderate loss of BMD during the first year of treatment.

CONCLUSIONS

There is a growing awareness of osteoporosis and its economic and social costs. The value of bone mineral density measures in fracture risk prediction is as good or better than the ability of blood pressure to predict stroke or serum cholesterol to predict heart attack. Currently, DXA is the most widely used technique for bone mineral assessment. There is an increased momentum to establish the role of peripheral techniques such as QUS and pDXA due to their low costs and potentially wide availability. Increased use of BMD measurements is also driven by the availability of effective and acceptable therapies for osteoporosis. Clinical questions, availability, and cost should govern the choice of site, number of sites, and the technique. BMD or ultrasound should not be used in isolation in the assessment of osteoporosis, but should be combined with risk factors, medical history, physical examination, and other laboratory tests in overall patient management.

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6

Technical Aspects of Skeletal Assessment Using Quantitative Ultrasound

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CONTENTS

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INTRODUCTION AND OVERVIEW OF QUANTITATIVE ULTRASOUND (QUS) IN BONE DENSITOMETRY

Ultrasound was used to evaluate the acoustic properties of bone as early as the 1950s (1–3). However, enthusiasm for the application of ultrasound measurements as a clinical tool to assess skeletal status developed only after the pioneering work of Langton and colleagues in 1984 (4), who reported that broadband ultrasound attenuation (BUA) measurements in the calcaneus discriminated between elderly women who had sustained a hip fracture and those with no history of fracture. In the nearly two decades since this initial report, there have been major advances in the use of ultrasound for determining skeletal status. Quantitative ultrasound (QUS) devices are now approved by the FDA and equivalent regulatory agencies throughout the world for clinical use in the evaluation of osteoporosis and prediction of fracture risk. As a result of data from large, prospective trials (5–8), there is now widespread consensus that QUS measurements are useful for assessing fracture risk in both elderly and peri-menopausal women (*see* Chapter 7).

This chapter provides an overview of the technical aspects underlying quantitative ultrasound measurements in bone. The text is divided broadly into several areas: an explanation of the basic aspects of ultrasound itself and ultrasound measurements in bone; an overview of clinical devices; a discussion of what ultrasound measurements reflect, as revealed by in vitro studies; and finally, technical considerations and chal-

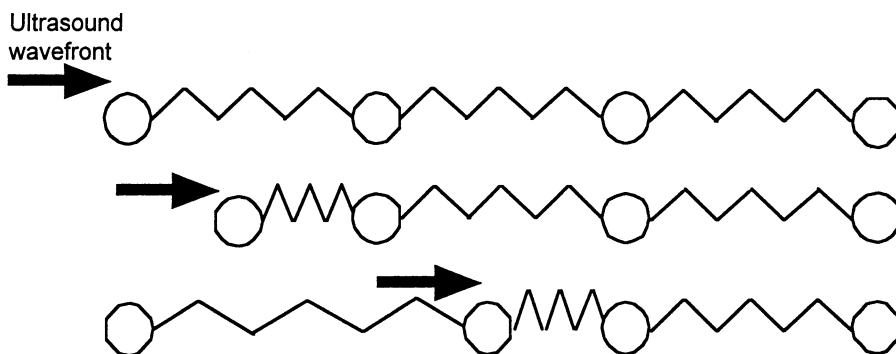


Fig. 1. Idealistic representation of sound propagating through particles connected with springs. For a longitudinal wave, the particles vibrate in the direction of the wave motion.

lenges for the clinical use and interpretation of ultrasound measurements. For additional information, the reader is referred to one of several excellent reviews of this technology and its clinical applications (9–15).

BASIC PRINCIPLES OF ULTRASOUND

What is Ultrasound?

A general appreciation of the physical nature of sound waves is essential to understanding QUS measurements in bone. Sound is generated by the oscillatory and vibratory motion of particles propagating in a fluid or solid medium. Unlike electromagnetic waves, sound cannot propagate in a vacuum. Propagation of sound occurs when the mechanical vibration invokes a tiny disturbance (or displacement) of the particles in the medium. Imagining that the particles in the medium are connected to each other by springs, the disturbance is transmitted in a stepwise fashion throughout the medium (Fig. 1). As such, the propagation of a wave depends on the intrinsic elastic properties (i.e., the springs) of the medium, and therefore, the speed at which the wave propagates (i.e., its velocity) is a characteristic of the medium. In a perfect fluid (gas or liquid), the particles in the medium can only undergo an oscillatory motion parallel to the direction in which the sound wave is travelling. The type of wave generated by this motion is called a *longitudinal* or *compression* wave. However, in solids, the particles in the medium may undergo an oscillatory motion perpendicular to the direction in which the sound wave is traveling. The type of wave generated by this motion is called a *transverse* or *shear* wave. Only longitudinal waves will propagate in soft tissues since they behave essentially like a viscous fluid. In comparison, both longitudinal and shear waves can propagate in a solid, such as bone.

The motion of an acoustic wave can be characterized in space and time by its frequency and wavelength. Frequency is defined as the number of cycles per second at which the particles in the medium vibrate, and is expressed in Hertz (Hz, 1/s). The wavelength represents the spatial excursion of a cycle of vibration (seen, for example, as the distance between two consecutive peaks). The following equation characterizes the relationship among sound velocity (c), frequency (f), and wavelength (λ):

$$\lambda = c/f \quad [\text{Eq. 1}]$$

In general, the human ear is sensitive to sound waves with frequencies between 20 Hz and 15–20 kHz. *Ultrasound* refers to sound waves with frequencies greater than 20 kHz.

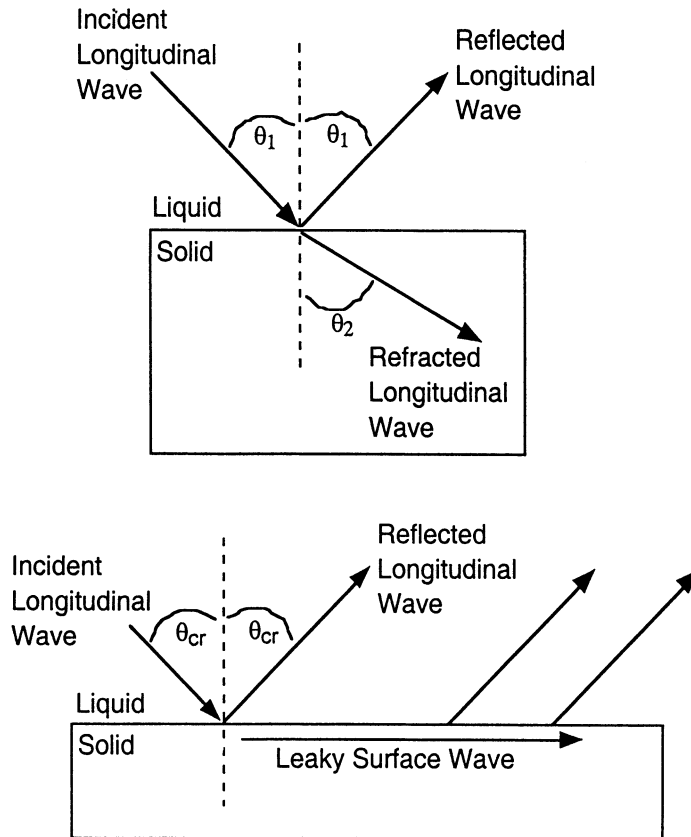


Fig. 2. Reflection and refraction of incident sound wave at the boundary of a liquid and solid media. The top figure shows the general case of a reflected and refracted wave. The bottom figure shows the special case where the incident wave strikes the surface at the critical angle, thereby exciting a leaky surface skimming wave. The Omnisense multi-site device takes advantage of this type of sound propagation phenomenon to perform velocity measurements.

Ultrasound has a number of clinical applications, including diagnostic imaging of soft-tissues and the developing fetus, as well as dynamic imaging of blood flow. In these applications, sound waves with frequencies ranging from 1–10 MHz are customarily employed. In comparison, due to the high attenuation of sound waves in cancellous bone, QUS applications generally employ much lower frequencies of 0.1–1 MHz. Thus, the corresponding wavelengths are typically 1–10 mm.

The generation of an ultrasound wave is most commonly accomplished using a piezo-electric transducer. This transducer employs special materials, often a type of ceramic, that convert an electrical signal into a mechanical vibration. In addition to generating an ultrasound wave, these transducers can also detect, or receive, a wave and therein convert a mechanical vibration into an electrical signal. Thus, these transducers are used to probe, or interrogate, a material with ultrasound.

In general, there are two basic approaches to probe materials using ultrasound. In the first approach, known as the reflection mode, a single transducer acts as both the transmitter and receiver. In this method, a portion of the ultrasound wave is reflected back to the transmitter whenever it encounters a change in the acoustic properties of the medium it is travelling in. This method is used to produce ultrasound images, such as those used in

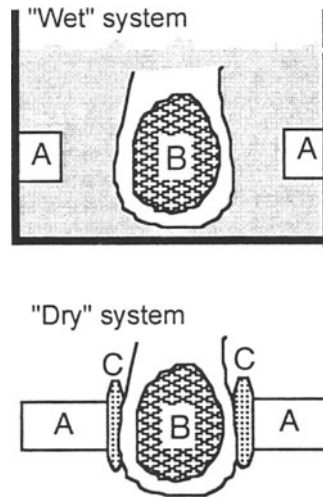


Fig. 3. Schematic diagram of quantitative ultrasound assessment of the calcaneus, where **A** represents the sending and receiving ultrasound transducer, **B** represents the calcaneus, and **C** represents the acoustic coupling gel. Systems using a water bath and gel for acoustic coupling are shown in the top and bottom figures, respectively.

monitoring fetal development. The second general approach, known as the transmission mode, uses two transducers, where one acts as a transmitter and the other as a receiver. The received signal is compared to a standard or reference waveform to deduce the acoustic properties of the material. The two approaches can also be used in combination. The reflection mode is most often used in medical imaging applications. It is slightly easier to implement as it requires only a single transducer, whereas the transmission mode requires two transducers along with access to both sides of the tissue being evaluated.

Transmission and Reflection of Sound Waves at Boundaries

With regard to the propagation of an ultrasound wave, it is important to recognize that as a sound wave passes from one material to another, some part of the wave will be reflected and some will be transmitted (Fig. 2). An example of this type of transition occurs as an ultrasound wave passes between soft tissue and bone. In general, the angles and intensities (i.e., the energy carried by an ultrasound wave) of the reflected vs transmitted signals can be related to the intensity of the incident wave according to the values of the *acoustic impedance* in the two materials. Acoustic impedance is a characteristic of a given media (or material), and is defined as the product of the density and sound speed in that given material. If two materials have identical acoustic impedances, then there will be no reflected wave. In general, the characteristic acoustic impedances of soft tissues are similar to that of water (Table 1). Because of the large impedance mismatch between soft tissue and air, the vast majority of the incident wave is reflected (and not transmitted). This mismatch in tissue impedance explains why ultrasound cannot penetrate air- or gas-filled organs, such as the lungs or bowel. In comparison, when an ultrasound wave passes from soft tissue to cortical bone, approx 25–50% of the energy of the incident wave will be transferred to the reflected wave, and 50–75% of the energy will be transferred to the transmitted wave.

Table 1
Typical Ultrasound Velocity, Acoustic Impedance, and Attenuation Values in Different Biologic Tissues for Temperatures in the Range Between 20° and 37°C (16)

<i>Tissue or media</i>	<i>Ultrasound propagation velocity (m/s)</i>	<i>Characteristic acoustic impedance ($\text{Ns}^{-1} \text{m}^{-3}$)</i>	<i>Slope of the ultrasound attenuation coefficient ($\text{dBcm}^{-1} \text{MHz}^{-1}$)</i>
Water	1480 ^a	$1.48 \cdot 10^6$	0.002 ^b
Air	340 ^a	440	—
Blood	1566	$1.66 \cdot 10^6$	0.2
Cancellous bone	1450–1800	—	10–40
Cortical Bone	3000–4000	$4.1\text{--}8.1 \cdot 10^6$	5
Fat	1450	$1.38 \cdot 10^6$	0.8
Liver	1560	$1.65 \cdot 10^6$	0.6–0.9
Muscle	1550–1630	$1.65\text{--}1.74 \cdot 10^6$	0.5–1.5
Skin	1600	—	2–4

^aAt 20°C.

^b0.002 $\text{dBcm}^{-1} \text{MHz}^{-2}$ due to the quadratic variation with frequency of attenuation in water.

This concept explains the importance of achieving good acoustic contact between ultrasound transducers and the skin, so that there are no air pockets to interfere with the wave transmission. In clinical QUS devices, this coupling is achieved by either submerging the foot in a water bath (for “wet” systems) or by applying gel between the transducer face and the skin (for “dry” systems). Thus, to decrease errors in clinical measurements it is critical to eliminate air bubbles in the water and on the skin for the water-bath systems, and to ensure a uniform application of gel between the transducer and skin surface for contact devices.

Attenuation of Sound Waves

Attenuation of sound wave as it propagates in a tissue occurs by a reduction in its amplitude, resulting in a loss of energy. The primary sources of wave attenuation include absorption, reflection, and scattering. *Absorption* losses convert the ultrasonic wave energy into heat. The conversion of acoustic energy into heat is a complex process that depends on the molecular composition of the material and the frequency of the ultrasound wave. In general, absorption increases with increasing frequency of the ultrasound wave. *Reflection* losses occur at the interface between tissues with mismatched acoustic impedances (see above). For example, reflection losses occur as a sound wave passes from water to skin, from skin to fat, and from muscle to bone. *Acoustic scattering* reduces the amplitude of the propagating wave as energy is redistributed in one or more directions. Scattering depends on the wavelength of the ultrasound signal and the specific characteristics of the tissue through which it is propagating. In general, scattering occurs as the propagating ultrasound wave encounters particles of different density than that of the surrounding medium. Thus, the particle oscillates differently than the surrounding medium, and a secondary wave is generated. The pattern of scattering can be quite complex in materials that are inhomogeneous, such as bone. Thus, it is likely that scattering contributes substantially to the total attenuation observed as an ultrasonic wave propagates through porous cancellous bone due to the large difference in density between the marrow and

trabecular bone network. During in vivo scans of the heel, the ultrasound wave encounters an even more inhomogeneous path, as it passes through skin, underlying soft tissue, cortical bone, and trabecular bone and marrow.

A more detailed explanation of the physics of ultrasound and ultrasound propagation in biologic tissues can be found in the recent review by Laugier (16).

PRINCIPLES OF ULTRASOUND APPLIED TO BONE

Overview of Clinical Ultrasound Devices

A variety of devices have been developed for use clinically to assess skeletal status (Table 2). Most of these devices utilize a transmission ultrasound approach. With this approach one transducer is used to send the ultrasound signal and a second transducer is used to receive the signal. The ultrasound transducers are acoustically coupled to the subject using either a water bath ("wet" systems) or a gel applied directly between the transducers and the skin ("dry" systems). In systems employing a water bath, the transducers are usually fixed a given distance apart. In contrast, for contact ultrasound systems, the transducers are moveable, so that they can be placed in contact with the skin (i.e., on either side of the calcaneus).

The skeletal sites that are measured by these devices vary, although the majority of systems available today assess the calcaneus. The precise techniques used to measure the parameters described below are specific to a particular device. However, the following sections will outline some general principles for assessment of acoustic parameters in bone. For additional information on specific clinical devices, one can refer to the recent reviews by Njeh and Blake (13), Cheng et al. (17), and Hans et al. (18).

Ultrasound Velocity

The velocity of an ultrasound wave depends on the properties of the medium through which it is passing, and the mode of propagation (i.e., longitudinal or shear wave). For a homogeneous, solid bar whose cross-sectional dimensions are small compared to the wavelength of the propagating wave, the velocity (v) can be related to the material properties of the bar by the following equation,

$$v = \sqrt{E/\rho} \quad [\text{Eq. 2}]$$

where ρ is the density of the medium (i.e., the bar), and E is the elastic modulus of the medium. This equation cannot be directly applied to ultrasound measurements in bone because bone, and cancellous bone in particular, is porous (i.e., is not solid), and has both marrow and bone elements (i.e., is not homogeneous). Therefore bone does not satisfy the conditions for which Eq. 2 was derived. Nevertheless, this relationship has been used in in vitro studies to develop empirical relationships between velocity, elastic modulus, and density (19–21).

A number of methods are available for measuring ultrasound velocity (22). Measurement of ultrasound velocity in bone is generally performed using a transmission technique. In this approach, the ultrasound velocity is computed by dividing the propagation distance (ie, how far the wave traveled) by the transit time (i.e., how long it took for the wave to get from the sending to the receiving transducer). Currently there is no accepted standard for the computation procedures or terminology. Thus, speed of sound (SOS), ultrasound transmission velocity (UTV), apparent velocity of ultrasound (AVU), and

Table 2
Devices for Performing Transmission QUS Measurements at the Calcaneus

<i>Device</i>	<i>Manufacturer</i>	<i>Coupling Medium</i>	<i>Parameters reported</i>	<i>Short-term precision, CV (%)^a</i>	<i>Standardized precision, SCV (%)^b</i>
Achilles	GE/Lunar (USA)	Water	BUA	0.8–2.5	5.7
			SOS	0.2–0.4	4.3
			Stiffness	1.0–2.0	2.8
DTU-One	Osteometer (Denmark)	Water (imaging)	BUA	1.0–1.4	NA
UBIS 5000	DMS (France)	Water (imaging)	BUA	0.8–2.5	3.3
			SOS	0.2–0.4	5.4
			BUB		
AOS-100	Aloka (Japan)	Gel	SOS	0.3–0.4	NA
			TI	0.8–1.5	
			OSI	1.2–1.8	
CUBAClinical	McCue (UK)	Gel	BUA	0.2–0.6	5.4–6.1
			SOS	1.5–4.0	4.8–5.4
Paris	Norland (USA)	Gel	BUA	1.8	NA
			SOS	0.4	
QUS-2	Quidel Corp (USA)	Gel	BUA	<1.5	NA
Sahara	Hologic (USA)	Gel	BUA	0.8–2.5	5.0–8.7
			SOS	0.2–0.4	3.0–5.7
			QUI	1.0–2.0	
			Est BMD	1.5–2.5	

^aShort-term precision, expressed as a coefficient of variation for repeat measurements, reported in the literature and summarized by Blake et al. (36), Njeh and Blake (13), and Cheng et al. (39).

^bStandardized coefficient of variation (SCV, %) computed as the CV/annual rate of change as summarized by Njeh and Blake (13), and Cheng et al. (39).

velocity of sound (VOS) are refer to essentially the same characteristic, although they may each be computed differently.

For transmission measurements in the calcaneus, three different methods have been used to compute ultrasonic velocity: time-of-flight velocity, heel (or limb) velocity, and bone velocity. Time-of-flight (TOF) velocity measurements are generally employed by QUS devices that use a fixed transducer separation. The TOF method measures the wave's transit time in the water-bath with and without the specimen, where the distance the wave traveled is determined by the transducer separation. Thus, the TOF velocity method assumes a constant heel thickness, and therefore the measured velocity is influenced by the actual heel width (23,24). As such, TOF velocity measurements must be interpreted carefully in studies where subjects have large differences in foot size, or where follow-up measurements are made in subjects whose foot size may change (due to growth, for example).

Both heel velocity and bone velocity measurements account for the width of the heel or calcaneus, respectively, in the calculations. Heel velocity and bone velocity measurements are typically employed by devices in which the transducers contact the skin. However, heel velocity measurements may also be calculated by QUS systems with a

fixed transducer separation by assuming a constant heel width for all subjects. A heel velocity measurement is calculated by dividing the overall heel width by the transit time, whereas the bone velocity measurement is calculated by dividing the calcaneus width by the transit time. While a distinction is made between these three methods for estimating ultrasound velocity in the calcaneus, in practice, the techniques yield slightly different values that are correlated strongly ($r = 0.83\text{--}0.98$) with each other (25). In general, TOF velocity measurements have the best precision, however heel and bone velocity measurements have a larger dynamic range, therefore increasing their sensitivity to detect longitudinal changes or differences between groups. Miller and colleagues (25) recommended that TOF velocity measurements should be performed for water-bath systems, whereas contact (“dry”) systems should use heel velocity measurements. In addition to velocity measurements at the calcaneus, ultrasound transmission techniques have also been used for ultrasound velocity measurements at the distal metaphysis of the phalanges (26,27). In this case, the transmitting and receiving transducers are placed on opposite sides of the phalanx, and the system computes an “amplitude-dependent speed of sound” (AD-SoS). The transmission methods described above may only be applied to peripheral skeletal sites, since too much of the wave’s energy would be attenuated by measurements at axial skeletal sites.

The semi-reflection technique is another method used to estimate sound velocity in bone. In this technique, an ultrasound wave which strikes the bone surface at a particular angle will generate a surface wave in the bone which propagates *along* the bone surface, rather than *across* the bone. The velocity can then be measured by placing transducers along the bone surface and measuring the transit time of the surface wave that is generated (Fig. 4).

In general, ultrasound velocity measurements at the heel range from 1400–2000 m/s, with heel and bone velocity measurements higher than time-of-flight measurements (25,28–30). Transmission ultrasound velocity (AD-SoS) measurements at the distal phalanx range from approx 1900–2300 m/s (31). Ultrasound velocities assessed by axial transmission at the mid-tibia, distal radius, phalanges, and metatarsals are typically higher, ranging from 2900–4300 m/s (32–35).

Broadband Ultrasound Attenuation

The attenuation-related parameter which has been most widely used in clinical studies is broadband ultrasound attenuation (BUA), which was first proposed by Langton et al. (4). BUA measurements are presently applied only at the calcaneus, and are a measure of the frequency dependence of the attenuation of ultrasound. Langton et al. found that the relationship between frequency and attenuation was nearly linear over the frequency range of approx 200–600 kHz in humans. Thus, BUA is defined as the slope of this frequency vs attenuation curve, and is reported in units of dB/MHz (Fig. 5). In contrast to ultrasound velocity, there are no theoretical relationships between ultrasound attenuation and the mechanical properties (such as elastic modulus) of a material. There are, however, a number of empirical observations relating BUA to the density, architecture, and mechanical properties of cancellous bone (20,21).

Other Acoustic Parameters

Commercial QUS systems that measure the calcaneus usually assess both BUA and velocity. In an attempt to reduce random measurement errors and perhaps improve

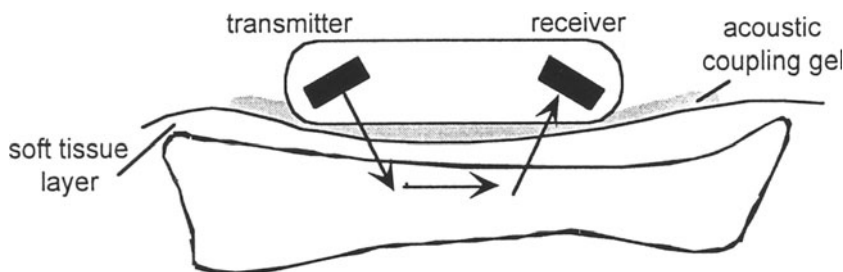


Fig. 4. Schematic diagram of the semi-reflection technique for estimating sound velocity in bone. In this technique, an ultrasound wave that strikes the bone surface at a particular angle will generate a surface wave in the bone which propagates along the bone surface, rather than across the bone. Thus, the velocity is measured by placing transducers along the bone surface at a known distance apart.

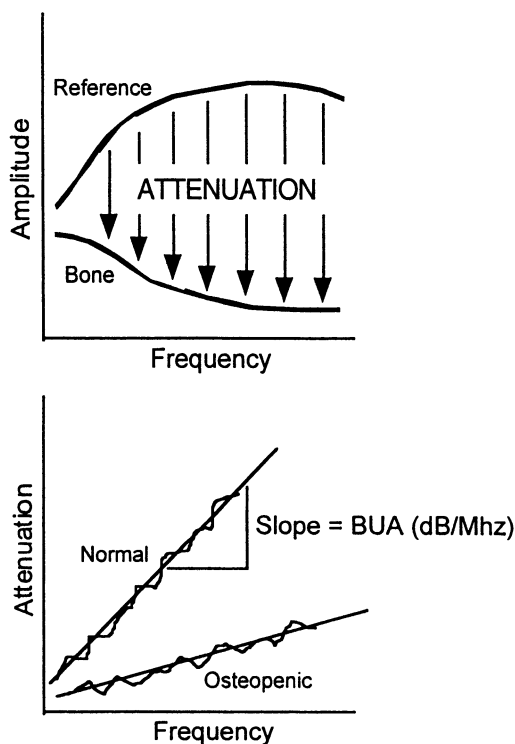


Fig. 5. Schematic representation of BUA measurement. The amplitude vs frequency curve is established for a reference material and then compared to that measured for the bone specimen to determine the frequency dependent attenuation. The slope of the frequency vs attenuation curve is defined as BUA.

precision and increase diagnostic accuracy, single parameters combining BUA and velocity have been employed. Although there is no theoretical acoustic-related basis for combining parameters, in practice, interpretation of scans may be easier for individuals less familiar with QUS measurements. The first example of a combined parameter was implemented on the Achilles (GE-Lunar, Madison, WI) system, and was called “stiffness.” It should be noted that the term ‘stiffness’ (defined below) has no particular

physical meaning, and is not related to the mechanical behavior of bone termed stiffness. In addition to the simplification provided by a single parameter, in the water-bath system, stiffness measurements are likely to converge to a stable value faster and be less sensitive to temperature variations than either BUA or SOS measurements alone (36).

$$\text{Stiffness} = 0.67 \cdot \text{BUA} + 0.28 \cdot \text{SOS} - 420 \quad [\text{Eq. 3}]$$

A combination parameter, defined as the quantitative ultrasound index (QUI) is also implemented in the Sahara bone sonometer (Hologic, Bedford, MA).

$$\text{QUI} = 0.41 \cdot (\text{BUA} + \text{SOS}) - 571 \quad [\text{Eq. 4}]$$

QUI correlates strongly with BMD at the heel, and thus has been used to provide an estimated BMD value that may be useful for prediction of fracture risk. A third commercial device, the AOS-100 (Aloka, Japan) also reports a parameter, osteo-sono-assessment index (OSI), which is a combination of SOS and BUA. Data from prospective cohort and clinical trials are needed to ultimately assess the clinical utility of these combined parameters.

DESCRIPTION OF CLINICAL QUS SYSTEMS

Calcaneus Measurements: Water-Coupled Devices

ACHILLES+ (GE-LUNAR CORP, MADISON, WI)

The Achilles+ ultrasound device consists of two unfocused 25-mm diameter transducers mounted co-axially at a fixed separation of 95 mm. Acoustic coupling is achieved through a temperature controlled water bath with pre-measured surfactant. The device measures BUA and velocity. Velocity is calculated using the time-of-flight substitution method. An estimated heel velocity is computed by assuming a constant heel width of 40 mm. An additional parameter, 'stiffness' is derived from the BUA and velocity measurements. Measurement time is approx 3–4 min.

DTU-ONE (OSTEOMETER MEDITECH, LOS ANGELES, CA)

The DTUone consists of two focused 20 mm diameter transducers mounted co-axially a fixed distance apart. The transducers are moved in a rectilinear pattern to generate an image of the calcaneus that is approximately 60 × 80 mm with a pixel size of 0.5 mm. Acoustic coupling is achieved through a room-temperature water bath. The device measures both BUA and SOS at each scan position. An automated algorithm chooses a region of interest for BUA and SOS measurements in an area of the posterior body of the calcaneus characterized by a local minimum of attenuation. Measurement time is approx 3–4 min.

UBIS 5000 (DIAGNOSTIC MEDICAL SYSTEMS, PEROLS, FRANCE)

The UBIS 5000 consists of two focused 29 mm diameter transducers mounted co-axially at a fixed separation of 100 mm. The UBIS system, like the DTUone, generates an image of the calcaneus using BUA data. To image the calcaneus the transducers are moved rectilinearly in 1 mm increments. The typical scan area is 60 × 60 mm, with a maximum scan area of 85 × 85 mm. The standard resolution for the BUA image is 1 mm per pixel. The device measures both BUA and velocity at each scan position. An automated algorithm places a circular region of interest (~ 1 cm²) in a region of the mid-

posterior calcaneus characterized by low attenuation (37). The location of the region of interest can be adjusted by the operator. A unique aspect is that the UBIS 5000 measures phase velocity, rather than group velocity. As phase velocity is strongly dependent on frequency, the manufacturer defines an ultrasound bone velocity (UBV) as the average phase velocity in the frequency range of 200–600 kHz (38). BUA, UBV, and broadband ultrasound backscatter (BUB) are computed for this region of interest. Measurement time is approx 1–2 min.

Calcaneus Measurements: Gel-Coupled Devices

ACOUSTIC OSTEO SCREENER AOS-100 (ALOKA CO. LTD, TOKYO, JAPAN)

The AOS-100 device consists of two 25 mm diameter transducers, encased in a soft rubber material, that are manually positioned over the calcaneus in direct contact with the patient's skin. Acoustic coupling is achieved using a gel. The device calculates a heel velocity, and a transmission index (TI), which reflects frequency-dependent attenuation in bone. In addition, a derived index, osteo sono-assessment index (OSI), is calculated from the SOS and TI measurements.

CUBA CLINICAL (McCUE PLC, HAMPSHIRE, UK)

The CUBAclinical device consists of two unfocused 19-mm diameter transducers mounted co-axially. Distance between the transducers is measured using a linear transducer, and constant contact pressure with the skin is maintained by springs. The transducers are mounted to silicone pads, and acoustic coupling is achieved using a water-based gel. The device measures both BUA and velocity. The velocity measurement (VOS) is a "heel velocity" as it accounts for the individual patients' heel width. Measurement time is approx 2 min.

PARIS ULTRASOUND (NORLAND CORP, FORT ATKINSON, WI)

The Paris ultrasound device consists of two unfocused 23-mm diameter transducers a fixed distance apart. Acoustic coupling is achieved by application of a water-based gel, in combination with a self-contained water system used to establish contact with the skin for various foot sizes and shapes. Using a manual pump, the water is drawn into a bladder from a water reservoir contained within the transducer housings. The device measures both BUA and velocity (VOS), and also computes an index, termed "soundness" that is a linear combination of BUA and VOS. The temperature of the water is measured and used to correct velocity measurements for temperature variability.

QUS-2 ULTRASONOMETER (QUIDEL CORP, SAN DIEGO, CA)

The QUS-2 ultrasonometer is unique in that it is one of the smallest QUS devices and in that it is a contact system wherein the transducers are moved over the surface of the heel to generate a measurement. The QUS-2 consists of two 16-mm diameter point source transducers with wide transmission and acceptance angles. In addition, these transducers have a hemispherical contact surface (in contrast to the usual flat-surfaced collimated transducers) that allows the QUS-2 transducers to move smoothly over the skin surface of the heel without losing acoustic contact (39). Acoustic coupling is achieved by application of a water-based gel. The total scanning area is approx 1400 mm², and is chosen to include the posterior and inferior surfaces of the heel. BUA, which is calculated using proprietary algorithms that produce a very stable measurement, is cal-

culated for a 70 mm² region of interest in the attenuation area of the calcaneus located for each individual based on the location and shape of the posterior and inferior surfaces of the calcaneus. Scanning time is approx 1–2 min.

SAHARA CLINICAL BONE SONOMETER (HOLOGIC, INC., BEDFORD, MA)

The Sahara clinical bone sonometer consists of two unfocussed 19 mm diameter transducers mounted co-axially on a motorized caliper. One transducer acts as a transmitter and the other as a receiver. The transducers are connected to semi-soft rubber pads, and acoustic coupling is achieved using a petroleum-based gel. The device measures BUA and SOS at a fixed region of interest in the mid-to-posterior body of the calcaneus. The velocity measurement (SOS) is a “heel velocity” as it accounts for the individual patients’ heel width. Two additional parameters are derived from BUA and SOS measurements: the quantitative ultrasound index (QUI) and estimated heel BMD. Measurement time is approx 10–30 s.

Finger Phalangeal Measurements

DBM SONIC 1200 (IGEA SRL, CARPI, ITALY)

The DBM Sonic device uses a fixed-point transmission technique to measure amplitude-dependent ultrasound velocity (Ad-SOS) in the distal metaphysis of the finger phalanges. The device employs two 12 mm diameter unfocused transducers affixed to a high precision caliper that measures the distance between the transducers. The transducers are positioned on the medio-lateral surfaces of the distal metaphysis of the phalanx using the head of the phalanx as an anatomic reference. Positioning of the ultrasonic probes is adjusted slightly until the optimum signal is visualized on the computer screen. Acoustic coupling is achieved by a ultrasonic gel. Measurements are conducted on each phalanx, and the results averaged. The short-term precision (coefficient of variation, CV) for Ad-SOS ranges from 0.3–1.1%, and the standardized coefficient of variation (CV/annual rate of change) is approx 2.0 (18). Measurement time is approx 5 min.

Multisite Measurements

OMNISENSE (SUNLIGHT ULTRASOUND TECHNOLOGIES, LTD., REHOVOT, ISRAEL)

The Sunlight Omnisense device employs a different methodology than standard transmission ultrasound devices. This device uses a semi-reflection technique to measure ultrasound velocity at various skeletal sites, including the one-third and ultradistal distal radius, phalanx, metacarpal, tibia, calcaneus, and posterior spinous processes. (It should be noted that among these sites, only the distal radius is approved in the US for clinical evaluations.) The measurement of ultrasound velocity utilizes proprietary transducers and measurement sequences that account for the soft tissue characteristics at the site of interest. In general the methodology is based on critical angle propagation chronometry and knowledge of how ultrasound signals are transmitted in bone. Essentially, as the bone surface is probed with an ultrasound wave, the wave is reflected and refracted in different directions within the bone. Part of the wave travels along the surface of the bone, and can be measured at a distance from the original transducer that emitted the probing wave (Fig. 4). The ultrasound velocity that is measured may reflect either cortical or cancellous bone properties, depending on the cortical thickness at the given skeletal site (18). The technique relies on the knowledge that ultrasound waves propa-

gate more quickly in dense tissue (such as cortical bone) than in soft tissues. For example, the ultrasound velocity in soft tissues ranges from 1520–1550 m/s, whereas velocity in cortical and cancellous bone ranges from 3000–4500 m/s and 1500–2400 m/s, respectively. Various arrays of transducers are used in different probes designed for the various skeletal sites.

WHAT DOES QUS MEASURE? CLUES FROM IN VITRO STUDIES

A widely held belief asserts that QUS may be advantageous (or complementary) to X-ray absorptiometry measurements because it reflects characteristics of bone that are distinct from the assessments of mass and density provided by X-ray absorptiometry. X-ray-based absorptiometry measurements are influenced nearly exclusively by the amount of material in the path of the X-ray beam. In contrast, at least theoretically, the propagation of ultrasound—because it is a mechanical wave—is influenced by the structure *and* composition (i.e., density) of the medium.

Initially, the clinical observation that calcaneal QUS measurements were only moderately correlated to BMD (either at the calcaneus or at other skeletal sites) was used as evidence that QUS measurements reflect characteristics of bone independent of density. This argument is flawed for several reasons. First, the observation of a moderate correlation between BMD and QUS does not provide *direct* evidence that in vivo QUS measurements are independent of BMD. Rather, the lack of a strong correlation may simply be explained by errors in either of the measurement techniques. Second, more recent studies that had better control of error sources and more precise matching of the region of interest used for QUS and BMD assessments have reported correlations between QUS and BMD assessed at the same skeletal site as high as 0.8–0.9 (10).

However, clear evidence that QUS measurements have the potential to reflect trabecular architecture independent of density comes from the observation that QUS measurements vary when cubes of cancellous bone are assessed in different directions (Table 3). For example, Glüer et al. (40) evaluated cubic specimens of cancellous bone from the bovine proximal radius and found that BUA was approx 50% higher in the superior-inferior direction than in either of the other transverse directions (medio-lateral or antero-posterior). Nicholson and colleagues confirmed these observations of acoustic anisotropy in human cancellous bone specimens from the lumbar spine (41). In their study, both BUA and ultrasound velocity were highest when measured along the superior-inferior direction. Additional evidence that QUS is influenced by structure was provided by Strelitzki et al. (42), who investigated bone phantoms and showed that pore size can affect acoustic properties independently of porosity.

In contrast to the studies mentioned above, nearly all studies of ultrasound (assessed in a single direction) and trabecular architecture in the human calcaneus find little evidence that ultrasound reflects architecture independently of density (43–46).

In summary, it is clear that ultrasound measurements *can* reflect features of trabecular architecture in certain circumstances. However, it is unlikely that in practice current measurement techniques (single-axis transmission) provide useful information about trabecular structure that is independent of density. The following sections will review these studies in greater detail.

QUS vs Density

On first glance, relationships between ultrasound properties and bone density appear straightforward, as both velocity and BUA appear linearly related to bone density. Strong

Table 3
Directional Dependence of Acoustic Variables in Cancellous Bone Specimens^a

Author	Bone sample	Axis 1 (proximodistal)	Axis 2 (anteroposterior)	Axis 3 (mediolateral)
Velocity (m/s)				
Njeh et al. (59)	Bovine femur	2252 ± 170	2094 ± 155	2171 ± 148
Nicholson et al. (41)	Human vertebrae	2249 ± 124	1761 ± 119	1709 ± 114
Njeh et al. (58)	Human femur	2698 ± 226	2431 ± 297	2409 ± 291
Han et al. (78)	Human tibia	2215 ± 298	1805 ± 147	1728 ± 166
BUA (dB/MHz-cm)				
Glüer et al. (40)	Bovine radius	51.2 ± 19.1	35.5 ± 24.7	33.3 ± 20.4
Nicholson et al. (41)	Human vertebrae	34.0 ± 22.4	12.4 ± 7.5	10.5 ± 6.9
Han et al. (78)	Human tibia	16.3 ± 14.1	16.4 ± 8.0	12.7 ± 6.7

^aAdapted with permission from ref. 20.

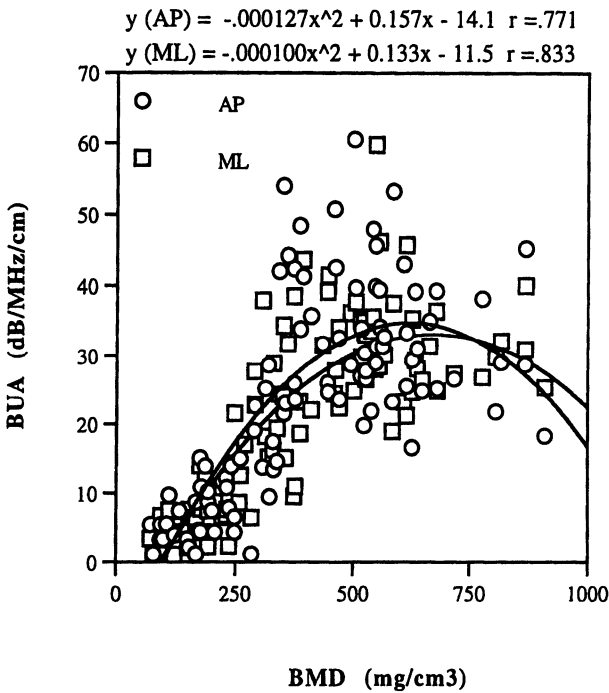


Fig. 6. Evidence of the nonlinear relationship between bone density and BUS. Reproduced with permission from ref. 49.

linear correlations between velocity and density ($r^2 = 0.83\text{--}0.88$), and BUA and density ($r^2 = 0.75\text{--}0.94$) have been reported (38,41). Despite these observations, it is now known that the relationships between BUA and density are more complicated.

Although linear relationships between BUA and density are consistently observed over *limited* density ranges, when evaluated over a large density range, BUA is a nonlinear function of density (Fig. 6) (47–49). Thus, in human cancellous bone specimens, there are strong positive relationships between BUA and density (41,47–51). Yet, in bovine cancellous bone, which is much more dense than human bone, relationships

between BUA and bone density are much weaker. Moreover, both positive and negative correlations have been reported for bovine bone (47–49,52–54). Taken together, these observations indicate that the underlying relationship between BUA and density is non-linear, whereby BUA and density rise together until porosities of about 60–75%, and then BUA falls as density increases (47–49).

These observations suggest that BUA is linearly related to bone density for the range of cancellous bone density values seen in osteopenic individuals. However, it suggests that ultrasound studies in bovine cancellous bone, or other species with high cancellous bone density, must be interpreted with caution, and in particular, their relevance to ultrasound behavior in human cancellous bone is questionable. The implications for clinical measurements must also be considered. It may be that in healthy young subjects, perhaps particularly in men, BUA may not be a reliable indicator of bone density.

In contrast to BUA, ultrasound velocity measurements appear to be linearly related to bone density for both human and bovine specimens over a wide range of density (38,41,48,49). In addition, demineralization studies in bovine cancellous bone over a wide density range support the notion of a linear relationship between velocity and density. However, it should be noted that velocity does vary with the orientation of the specimen, and therefore, it certainly may reflect other characteristics besides density.

QUS vs Trabecular Structure

In their review, Nicholson and Njeh (20) suggest that in Langton's initial paper describing the use of ultrasound to identify osteoporotic individuals, BUA was proposed as a surrogate for bone mineral density measurements, and the idea that bone structure could influence BUA measurements was not mentioned at that time. The notion that trabecular structure influences ultrasonic properties was implied later by McKelvie and Palmer (55), Langton (56), and Jones et al. (57), who observed qualitatively different relationships between BUA and bone density in cortical and cancellous bone.

ACOUSTIC ANISOTROPY IN CANCELLOUS BONE CUBES

In the first studies evaluating the influence of trabecular structure on acoustic properties, qualitative assessments of structure were employed. Essentially, cubic specimens of cancellous bone were excised, and ultrasound measurements performed in each of the three perpendicular directions of the cube (40,41,58,59). In these studies of cancellous bone from both bovine and human sources, both BUA and velocity were influenced by the direction of the measurement (Table 3). Because density is constant, these observations imply that acoustic properties reflect trabecular bone structure.

In addition, by measuring changes in BUA following progressive crushing of bovine cancellous bone, Tavakoli and Evans (60) provided further qualitative evidence that ultrasound measurements are influenced by bone structure.

ULTRASOUND VS QUANTITATIVE MEASUREMENTS OF TRABECULAR STRUCTURE

In addition to the qualitative assessments of bone structure mentioned above, several studies have investigated the relationship between ultrasound and quantitative assessments of cancellous bone microstructure, such as trabecular thickness, trabecular number, trabecular separation, and connectivity (43,45,46,53,61–64). These studies are often hard to interpret because bone density, trabecular structure, and ultrasound parameters are all inter-dependent. This interdependence makes it difficult to use regression models to prove *causality* in the relationship between ultrasound and structure.

Table 4
Correlation (r) Between Ultrasound Parameters and Trabecular Structure
in Human Cancellous Bone

	<i>Hans et al. (43), Calcaneus</i>		<i>Nicholson et al. (61), Vertebral Body</i>	
	<i>BUA</i>	<i>SOS</i>	<i>BUA</i>	<i>SOS</i>
BV/TV	0.78	0.79	0.72	0.67
Tb.Th	0.82	0.82	0.52	0.48
Tb.Sp	-0.70	-0.75	-0.74	-0.66
Tb.N	0.65	0.67	0.73	0.66

Glüer et al. (53) used micro-computed tomography with an approximate spatial resolution of 80 μ to evaluate structural parameters in bovine cancellous bone. They reported that a number of significant correlations between QUS and structural parameters, including some that were present even after adjusting for bone density. However, this study must be interpreted with caution due to the use of high-density bovine cancellous bone that may interact differently with ultrasound than human cancellous bone does. For example, the mean trabecular thickness and separation for these bovine specimens was 188 and 441 μ , respectively. In comparison, trabecular thickness and separation in human cancellous bone from the calcaneus are approx 85 and 900 μ , respectively (43).

In general, studies using human cancellous bone specimens find that ultrasound measurements are moderately correlated with trabecular structure (Table 4). However, when the regressions are adjusted for density, few, if any of the structural parameters remain independent predictors of the ultrasound properties (43,45,46,61–64). For example, Hans et al. (43) performed QUS measurements on cancellous bone specimens from human calcanei, and then performed two-dimensional histomorphometric measurements. They concluded essentially that ultrasound measurements reflected bone quantity rather than microarchitecture. Similarly, Nicholson and colleagues (64) performed QUS and microcomputed tomography measurements on cancellous bone specimens from 48 human calcanei. They found that bone volume fraction explained 93–95% of the variability in ultrasound measurements, with an additional contribution of, at best, 4% attributable uniquely to trabecular architectural features. In total, these studies suggest strongly that ultrasound measurements in a single direction essentially reflect bone density. Moreover, because bone density and microarchitecture are so strongly inter-related, it is unlikely that ultrasound measurements in a single direction reflect bone structure independently of bone density.

QUS vs Mechanical Properties of Bone

ULTRASONIC MEASUREMENTS AND THE MECHANICAL PROPERTIES OF EXCISED BONE SAMPLES

There are two possible approaches for predicting the mechanical properties of cancellous and cortical bone using ultrasonic measurements. In the first approach, a direct theoretical relationship between ultrasound velocity, elastic modulus, and density that was derived for solid homogeneous materials is employed. In the second approach, empirical relationships between ultrasound and mechanical properties are developed using regression models.

As mentioned previously, in a prismatic or rod-like solid specimen whose lateral dimensions are significantly less than the wavelength of the propagating ultrasound

wave, the wave travels at the *bar velocity* (v_{bar}). Under these specific conditions, there is a well-defined relationship between the bar velocity, Young's modulus of elasticity (E) and density (ρ):

$$v_{bar} = \sqrt{E/\rho} \quad [\text{Eq. 5}]$$

Thus, by measuring ultrasound velocity and density, the elastic modulus can be directly determined. Abendschein and Hyatt (65) measured the bar velocity at 100 kHz in human cortical bone specimens. They reported a strong linear correlation ($r = 0.91$) between the elastic moduli derived from ultrasonic measurements and those obtained from traditional mechanical testing methods. This approach has also been employed to assess the elastic modulus of extremely small, machined cortical bone specimens (crosssection = $300\ \mu$) and individual trabeculae, demonstrating a higher elastic modulus in cortical than trabecular bone tissue (21 vs 15 GPa, respectively) (66).

Ultrasound bar velocity measurements in cancellous bone were developed by Ashman and coworkers (67,68), who used low frequencies (50 kHz) to assess defatted human and bovine cancellous bone specimens. At these low frequencies, the wavelength was approx 20 mm, which was larger than the specimen's cross-sectional dimensions (~ 5 mm). They reported very strong correlations between ultrasonically and mechanically-derived elastic moduli ($r^2 = 0.93\text{--}0.96$). Other investigators have also used this technique to determine the elastic modulus of human cancellous bone from different skeletal sites (66,69–71).

When higher frequencies are employed the wavelength becomes much smaller than the size of the specimen, and ultrasound then travels at the *bulk wave* velocity. Under these conditions, there are also well-defined theoretical relationships between the wave velocity, density and the elastic properties of the propagating medium (58,72,73). These relationships, although similar in form, are more complex than the bar velocity/elastic modulus relationship. They have been used to evaluate anisotropy (i.e., directional dependence) in the material properties of cortical bone (70,74–76).

Because these theoretical relationships are not strictly applicable for the case of cancellous bone where fluid is present in the pores of the trabecular network (i.e., all in vivo measurements), a number of studies have derived empirical relationships between the mechanical behavior of bone specimens, bone density, and acoustic parameters (44,51,77–81). Investigators have employed both linear (78) and logarithmic (79) relationships to describe the relationship between ultrasonic parameters and mechanical properties, such as elastic modulus and ultimate strength.

Although there is no consensus, in general, velocity has been found to be a better predictor of the mechanical properties of cancellous bone than BUA. Studies conflict as to whether BUA or SOS are independent of density in their ability to predict mechanical properties. Some studies suggest that QUS parameters can add predictive power beyond that of density alone for estimation of mechanical properties of cancellous bone, although the additional amount of variance accounted for is usually small ($\sim 3\text{--}8\%$) (51,79,80,82). However, other studies report that QUS gives no better explanation of mechanical behavior than density alone (44,78). The discrepancies among these studies may relate to differences in experimental procedures, as some investigators performed measurements in the intact cadaveric heel (51), whereas the majority used only excised cancellous bone specimens. In addition, results may vary according to whether bovine or human bone was used.

Taken together, these data indicate that, at least in excised bone specimens, ultrasonic measurements can reflect mechanical properties. However, their ability to predict mechanical behavior independently of density is still controversial. It is unclear how well these in vitro studies can be extrapolated to clinical QUS measurements, as most of the in vitro studies were not performed at body temperature, and more importantly, the propagation of an ultrasound wave is far more complex in vivo. In transmission measurements of the calcaneus, for example, the ultrasound signal must pass through skin, subcutaneous tissue, the cortical shell, the cancellous bone and marrow, the second cortical shell, and more subcutaneous tissue and skin before reaching the receiving transducer.

ULTRASONIC MEASUREMENTS AND WHOLE BONE STRENGTH

Another empirical approach to understanding the potential role of ultrasound measurements in the prediction of bone fragility has been to compare QUS measurements to whole bone strength, either at the same site or at a distant skeletal site. In this regard, Han and colleagues (83) showed that BUA of the heel correlates moderately to strongly ($r=0.79$) with the strength of the calcaneus itself. Although heel QUS parameters are moderately correlated with femoral failure loads ($r^2=0.40-0.70$), in general, femoral BMD is the best predictor of femoral load ($r^2=0.71-0.92$) (84-87). In contrast to observations for femoral failure loads, heel QUS variables are only moderately to weakly correlated with vertebral failure loads (87,88). In comparison with hip and spine BMD, the weaker correlations between heel QUS and whole bone strength are consistent with their slightly lower predictive ability for fractures in vivo (5,6,89).

TECHNICAL CHALLENGES FOR SKELETAL ASSESSMENT USING QUS

As mentioned earlier, the ability of QUS to predict fracture risk has been well documented by several prospective clinical trials (5-8,90,91). Accordingly, the use of QUS in the clinical management of osteoporosis is growing. Yet, in general, QUS devices have not achieved widespread acceptance. This lack of widespread use may be attributed to several factors, including an undefined strategy for how best to use the measurements (92), a lack of standardized measurements and quality control procedures (11), concerns about the applicability of WHO diagnostic criteria (93,94), and reduced precision due to a variety of possible error sources in the measurements (95). Thus, for QUS measurements to achieve wider clinical acceptance, each of these issues must be addressed.

Standardization, Quality Assurance, and Quality Control

As mentioned previously, because QUS assessment of bone is not yet standardized, the techniques, algorithms, and terminology employed varies with each device. As such, velocity and attenuation measurements made on different devices are not necessarily comparable. As such, it has proven difficult to compare the performance of QUS devices across different study populations.

In clinical applications, quality assurance (QA) refers to methods established to evaluate the performance of equipment, assays and operators with the goal of improving reliability. In comparison, quality control (QC) generally refers to a set of test procedures and checks designed to verify the quality of the final data. In both cases, standard assays or phantoms may be used for training and/or validation procedures. Adequate QA and QC procedures are particularly important for QUS assessment of skeletal status, as the

changes in acoustic parameters due to aging and/or treatment are relatively small ($\sim 1\text{--}5\%$ /yr). Thus, procedures to enhance the precision and reliability of QUS data will certainly improve its clinical utility.

In X-ray based bone densitometry, the most common QA method is the regular scanning of a standard phantom that mimics bone and soft tissue. Regular scanning and analysis of a standard and/or phantom can be used to monitor the performance of the system to ensure it is operating within specified guidelines. A “standard” test object generally has known acoustic properties similar to those measured in patients, and has a relatively simple shape/geometry. In comparison, a “phantom” generally mimics patient measurements both in terms of acoustic properties as well as physical shape and geometry. Presently there are no universal standards or phantoms that can be used effectively for all QUS devices. Therefore, for QC evaluation, each manufacturer supplies a reference standard with their device. The standards are generally composed of a uniform plastic or rubber material, in which ultrasound velocity is well characterized. Thus, the material can be chosen to reflect the velocity measured in patients. It is more difficult to characterize attenuation for a given reference standard, as it depends not only on the material comprising the standard, but also the geometry of the standard. As such, attenuation values for each reference standard must be established empirically. Design of a stable reference standard that reflects clinically-relevant values for BUA and SOS has proven difficult. Nonetheless, regular measurements of the manufacturer-supplied phantom are necessary to detect any changes in the device performance due to aging or failure of the components of the scanner. Measurements of the reference standard do not constitute a calibration of the device, but rather a check to ensure that the device is operating properly.

In addition to the reference standards provided by the manufacturers, there are two other reference standards that have been developed independently for evaluation of QUS device performance. The “*Leeds phantom*” consists of liquid epoxy and hardener mixed with cubic granules of gelatin. After hardening of the epoxy during production, the gelatin is removed, the resulting pores filled with sunflower oil, and the standard is encased in a hollow Perspex cylinder. Several standards are available in order to cover the range of porosity (50–83%), velocity (1480–1650 m/s), and BUA (40–140 dB/MHz) values observed clinically (96,97). The “*Vancouver phantom*” consists of a reticulated vitreous carbon form filled with heavy grade USP oil (98). Like the Leeds phantom, there are several Vancouver phantoms available to span the range of clinically relevant BUA values (25–70 dB/MHz). However, the ultrasound velocity (1650 m/s) is independent of porosity, in contrast to what is seen clinically.

One QA/QC problem often encountered in clinical practice is the issue of cross-calibration of devices. Cross-calibration is necessary, for example, when an upgraded device is installed or when subjects are involved in multi-center trials that may or may not use the same device. Cross-calibration techniques are reasonably well established for X-ray based bone densitometry (99–101). In contrast, due to the absence of standardized QUS parameters and the lack of a universal reference phantom, methods for cross-calibration of QUS devices have not been established. Strelitzki and colleagues (96) measured acoustic phantoms on three different QUS devices and found that variations in acoustic parameters between different devices as well as between nominally identical devices from the same manufacturer were of sufficient magnitude to be clinically relevant. They strongly suggested the development of universal quality standards and adaptation of consensus defi-

nitions for terminology and techniques. To study the performance and comparability of different devices, Njeh et al. performed QUS measurements in 35 women who had recently suffered a hip fracture and 35 age-matched controls using six calcaneal QUS devices, three gel-coupled and three water-coupled (102). They reported moderate to strong correlations between SOS ($r = 0.79\text{--}0.93$) and BUA ($r = 0.71\text{--}0.92$) measurements on the six different devices. However, confirming previous reports, the absolute values for BUA and SOS varied significantly between devices. The mean BUA and SOS values ranged from 53–101 dB/MHz, and 1490–1600 m/s, respectively.

These findings confirm that, in general, QUS measurements are not comparable across different devices. Presently, there is no accepted standard terminology or methodology for acoustic evaluation of skeletal status. Further research is required to establish universal reference phantoms, and to evaluate the effectiveness of these phantoms for cross-calibration of QUS devices.

Error Sources

In general, the clinical accuracy of a bone densitometry method determines its ability to identify those individuals at increased risk of fracture, whereas the precision of a method determines its ability to monitor age-, disease- and treatment-related changes. However, clinical accuracy and precision are not separable, as a method which has poor reproducibility will have reduced diagnostic sensitivity (i.e., diagnostic accuracy) due to the introduction of random error into the measurements. Barkmann and Glüer (95) have identified a number of error sources that may affect the accuracy and precision of QUS measurements. Error sources for QUS measurements can be broadly divided into those that are associated with the individual subject or those that are associated with the technique itself.

Error sources that can be associated with an individual include variability in bone size, soft tissue characteristics, bone marrow composition, and distribution of cortical versus cancellous bone at a given skeletal site. Error sources that are associated with the technique itself include equipment error or drift, positioning, efficiency of acoustic coupling, and limb, waterbath and ambient temperature. Moreover, in ultrasound devices that use water for acoustic coupling, the immersion time of the foot and the concentration and nature of wetting agents are potential sources of variability. In general, as discussed in the previous section, equipment drift and/or failure can be detected by regular scanning of standard reference phantoms.

EFFECT OF BONE SIZE AND SOFT TISSUE PROPERTIES

Intersubject variability in bone width and cortical thickness has been shown to influence QUS measurements (23,24,103–106). For example, Wu et al. (105) observed a nonlinear relationship between BUA and bone width in vitro, and others have reported an association between heel width and SOS measurements in vivo (23,24). Obviously the errors associated with variations in bone size are likely to have the greatest impact on the interpretation of QUS measurements in growing children or in individuals with differing body sizes.

Changes in the soft tissues surrounding the bone, such as those due to alterations in body weight and/or retention of fluid at the measurement site, can also influence QUS measurements. Using the Achilles commercial device, Kotzki and colleagues (24) reported that increasing thickness of fat around cadaveric heels decreased SOS measure-

ments, but did not consistently affect BUA. Johansen and Stone (107) studied the effect of ankle edema on QUS measurements at the calcaneus using a contact device. They found that a decrease in heel width of 6 mm (by pressure to disperse the edema-related fluid) led to reductions in SOS and BUA that were equivalent to approximately one-quarter of a standard deviation of the reference range.

EFFECT OF POSITIONING

It is important to note that with regard to heel QUS systems, the calcaneus itself exhibits significant heterogeneity both with respect to its external surface geometry as well as the distribution of mass within the cortex (108–110). Due to this heterogeneity, ultrasound attenuation may vary as much as 30% within an individual, depending on the region of the calcaneus being assessed (111–113). This heterogeneity contributes to the potential errors associated with use of a fixed region of interest. Indeed, several investigators have demonstrated that acoustic parameters are strongly influenced by the location of the region being evaluated as well as the position of the foot with respect to the ultrasound probes (37,111,114,115).

For QUS devices that employ fixed transducers, and thus a standard region of interest, interindividual differences in bone size and soft tissue thickness can influence the location of the region that is evaluated (116). The use of a fixed ROI is likely to be most problematic in longitudinal evaluations of growing children and in cross-sectional comparisons of individuals with varied skeletal size, such as men and women. The use of QUS devices that provide an image of the calcaneus may reduce these errors by allowing either automated placement of the ROI or operator choice of the placement of the ROI. For example, a region of low density and low attenuation can be found in the greater tuberosity of the calcaneus and used to define an ROI for evaluation (117). Jorgensen et al. (118) used a single device to show improved precision for BUA measured using this anatomically-defined ROI (located by an imaging QUS system), compared to values derived from a fixed ROI (CV=1.2% vs 3.8%, respectively). Similarly, Frost and colleagues (119) compared precision of two heel water-bath QUS devices and found that the standardized precision of BUA and SOS measurements was better for an imaging system with automated ROI placement than for a nonimaging device with a fixed ROI. However, they also reported that the imaging system did not provide improved long-term precision when compared to the device employing a fixed ROI, and moreover, that both systems had similar abilities to discriminate individuals with vertebral fractures from healthy, unfractured control subjects. Thus, while the idea of an imaging system is attractive, additional studies are required to test whether imaging capabilities will lead to more accurate measurements, enhanced diagnostic sensitivity, and better fracture risk prediction.

Another potential error source related to positioning is the variability in foot position within the device. Variability in foot position can occur as mediolateral displacement, superior-to-inferior displacement, heel-to-toe displacement, or as a rotation about the long axis of the leg. For example, superior and inferior displacement of the optimal region of interest by 5–20 mm results in up to a 3% variation in SOS and an 8% variation in BUA values (111). Furthermore, rotation of the foot by just 2.5° causes up to a 2–4% change in BUA (114,115). Whereas QUS devices with capabilities for imaging and flexible ROI placement can potentially reduce errors associated with use of a fixed ROI and heel-to-toe and superior-inferior displacements, they cannot compensate for variability in foot rotation (113,115,116,118,119).

QUS measurements using other devices and/or other skeletal sites are also subject to positioning-related errors. For example, SOS measurements of the phalanges are based on the pattern of the received ultrasound signal. The optimal pattern is achieved by as the operator moves the transducers manually along the finger to achieve the best signal. Thus, the precision depends on the attention, experience and motivation of the operator.

FACTORS INFLUENCING ACOUSTIC COUPLING

Recall that acoustic coupling may be achieved either through use of a water-bath or through use of a gel applied directly between the transducer and the skin surface. In either case, air bubbles in the sound path contribute significantly to error in QUS measurements. Procedures to reduce the collection of tiny air bubbles on the skin surface, such as scrubbing the heel or use of a water jet directed at the skin, may improve measurement reliability (120).

Another potential error source associated exclusively with water-bath systems is that associated with immersion time of the foot (114,115). Chappard et al. (115) evaluated QUS values for ten consecutive measurements at 3-min intervals, with no repositioning of the foot. They showed that QUS values varied with the first few scans, but then reached relatively stable values after the first two of three scans for BUA and after the sixth or seventh scan for SOS. The variation in QUS values with immersion time was most pronounced for BUA measurements in postmenopausal women, where a 21% difference between the first and tenth scan was observed. Thus, immersion time should be closely monitored to ensure repeatable scan data.

INFLUENCE OF TEMPERATURE

Acoustic properties of many biological tissues are temperature-dependent. Ultrasound velocity in fat and fatty tissues decreases with increasing temperature, whereas water, blood and muscle show the opposite trend (121). In vitro studies of cortical bone indicate that velocity decreases with increasing temperature at approx 6 m/s/°C (122). It is therefore likely that QUS measurements in vivo will be affected by temperature. Accordingly, it has been shown that both the ambient temperature of the room, as well as the temperature of the water bath and the core temperature of the foot can influence QUS measurements (123–126).

We performed QUS measurements in human cadaveric feet at controlled temperatures in the calcaneus itself ranging from 25–49°C, and found that ultrasound velocity decreases linearly with increasing temperature (–1.8–2.8 m/s/°C) and BUA increases with increasing temperature (0.21–0.75 dB/MHz/°C) (124). Pocock and colleagues had similar findings in their study of a single cadaveric foot (126). The negative thermal coefficient for velocity is likely due to the influence of fat in the marrow of the calcaneus. Thus, a different response to temperature may be observed in QUS systems that use the semireflection technique to assess ultrasound velocity in cortical bone with little to no fat content. Overall, however, this finding implies that QUS measurements in patients with a greater proportion of fatty marrow (i.e., those with low bone mass, perhaps) would tend to be most sensitive to temperature-related changes in velocity. Iki and colleagues (125) showed that the room temperature may also affect QUS measurements. They assessed the effect of season on QUS parameters in five healthy women, and found that SOS and stiffness measurements were significantly greater in February (room temperature = 12.6°C) than in June (room temperature = 22.4°C). Taken together, these in vitro

and in vivo observations indicate that controlling temperature may reduce QUS measurement errors.

In summary, these observations indicate that in order to achieve optimal accuracy and precision in QUS measurements, it is important to have a standardized measurement protocol with specific guidelines for monitoring immersion time, positioning, and temperature.

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7

Clinical Aspects of Skeletal Assessment with Ultrasound

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INTRODUCTION

Osteoporosis is common affecting nearly 28 million women and men in the United States. This is a major problem since the clinical consequence of osteoporosis, fracture, results in morbidity and mortality, and contributes to health care costs (1). The challenge for health care providers is to identify those individuals at risk prior to fracture.

Low bone mineral density (BMD) is the single best predictor of fracture risk. For every standard deviation decrease in BMD, fracture risk increases two- to threefold. This relationship is true whether BMD is measured at the axial or appendicular skeleton. Dual energy x-ray absorptiometry (DXA) is the preferred technique to quantitate BMD. However, patient access to the instrument is often limited resulting in failure to diagnose low bone mass. The use of lower cost, portable peripheral technologies has been suggested as a means to increase osteoporosis diagnosis before fracture (2). One such technique is quantitative ultrasound (QUS) (3–5).

QUANTITATIVE ULTRASOUND AS AN INDICATOR OF OSTEOPOROSIS

Factors Affecting QUS Values

Numerous studies have demonstrated that the QUS measurements (broadband ultrasound attenuation [BUA], speed of sound [SOS]), and the derived measurements (stiffness, quantitative ultrasound index [QUI], estimated BMD) decrease with age (6–14).

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The rate of bone loss is significant in postmenopausal women (6,12). In addition to age, years since menopause is a significant factor in determining ultrasound values (9,11). Factors that have an impact on DXA measurements also appear to impact the QUS measurements. Family history of osteoporosis, body weight, physical activity, and dietary calcium intake affect QUS values (13). In contrast, smoking, alcohol and coffee consumption, activities of daily living, oral contraception use, and traumatic fractures are not associated with QUS values (13). The strength of association of stature, physical activity, and protein and calcium intake for the QUS measurements is similar to that observed for DXA (14,15). QUS measurements are higher in African-Americans than Caucasians (15,16), but these differences are attenuated by adjustment for calcaneal BMD (16).

Local factors can also impact the QUS measurements. Ankle edema is associated with a significant decrease in both BUA and VOS (17), but calcaneal width has no impact unless marked variability is encountered, e.g., pediatric patients (18). In ultrasound systems using a water bath, increasing water temperature decreases QUS values (17). Thus, the precision of ultrasound instruments may be affected by temperature variation of the environment or of the patient's limb (19).

Correlation of QUS Values with DXA

In an attempt to demonstrate the utility of QUS to diagnose individuals with low bone mass, investigators have examined the relation between ultrasound values and site matched BMD as well as BMD measurements of the axial and appendicular skeleton. The observed correlation coefficients between QUS of the calcaneus and BMD of the calcaneus range between $r = 0.58$ and 0.8 (8,14,20–25). Although these correlations are high, they leave 35–60% of the variability unexplained. It remains unclear whether this variability is explained by the ability of QUS to measure some other properties of bone, e.g., structure and/or strength, or to some unrelated factor, e.g., precision of the QUS measurements. Similarly, the correlation coefficient between BUA and VOS of the calcaneus is 0.74 , indicating either that the two ultrasound values measure properties of bone different from each other or that the variability is independent of skeletal status (19).

The correlations between QUS measurements and BMD at the spine and hip are lower, ranging from $r = 0.3$ to 0.87 (7,8,10,16,22–35). This wide range possibly reflects the study of different patient populations of varying age and skeletal status. More likely, however, the discordance is a reflection of differences in age-related bone loss at various skeletal sites and technology-related differences (36). The implication of these observations is that measurement of bone mass at one skeletal site cannot be used to accurately estimate bone mass at a different site. This is not unique to correlations using different technologies. The correlations between BMD at the spine and BMD at the total hip or femoral neck by DXA are $r = 0.64$ and $r = 0.61$, respectively (37). The correlation between the two hip sites is better, $r = 0.88$, but variation remains. Thus, DXA of the spine does not predict with certainty DXA of the hip. Even DXA of the total hip does not predict DXA of the femoral neck with certainty. In the same study population, the correlation between estimated BMD at the calcaneus determined by QUS had correlations of 0.44 – 0.50 with axial BMD (Table 1). Thus, ultrasound assessment of the appendicular skeleton will not predict with certainty BMD at axial sites. Recently, QUS imaging devices have been studied to determine if the correlations with axial BMD can be improved. The initial results indicate that the use of imaging QUS devices does not significantly improve the correlations between ultrasound values at the calcaneus and BMD at the spine and hip (24,35).

Table 1
Correlations Between QUS and DXA Measurements

	<i>QUS calcaneus</i>	<i>DXA spine</i>	<i>DXA femoral neck</i>	<i>DXA total hip</i>
QUS calcaneus	—	0.44	0.45	0.50
DXA spine	0.44	—	0.61	0.64
DXA femoral neck	0.45	0.61	—	0.88
DXA total hip	0.50	0.64	0.88	—

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Correlation of QUS with Biochemical Markers of Bone Turnover

Recent studies suggest that bone turnover as well as BMD is an important predictor of skeletal health (38). The relationship of QUS values to biochemical markers of bone turnover has been examined in several studies (39–41). In the early menopausal period, serum osteocalcin levels are significant determinants of the stiffness index (39). In the same time period, measurements of urinary collagen crosslinks are correlated with QUS measurements (40). However, the correlations are too low to allow prediction of QUS values from the values of bone turnover in the individual patient (39,40). Correlations between QUS values and biochemical markers or bone resorption in women with osteoporotic fractures range between $r = 0.55$ and 0.85 (41). Interestingly, there was no significant correlation between the markers of bone resorption and BMD of the spine or hip. The data suggest QUS measurements of bone may be influenced by bone turnover rate (39–41). However, the variations observed are such that neither measurement of QUS nor markers of bone turnover allow the prediction of the other in the individual patient.

Sensitivity and Specificity of QUS for the Diagnosis of Osteoporosis

The use of peripheral instruments that measure BMD has been proposed as a means to facilitate the diagnosis of osteoporosis (42). It is apparent that the QUS measurements are capable of differentiating groups of normal and osteoporotic women (7,8,10,16,22–35). However, the sensitivity and specificity of the instrument is the issue for the clinician who is evaluating a specific patient. The studies (37,43–50) suggest that the high false negative and high false positive rates limit the utility of QUS as the sole diagnostic technique on which to base therapeutic decisions.

Initial reports in a small group of women suggested that a BUA value of 63 dB/MHz had a sensitivity and specificity of 76% for the diagnosis of osteoporosis as the hip or spine (43). At a BUA of 72 dB/MHz the sensitivity was 93% and the specificity was 41% for the diagnosis of osteoporosis, but the sensitivity for fracture detection was 100% (43). Subsequent evaluations in larger cohorts of women suggest that QUS cannot be used to accurately diagnose the individual patient. In a study of 587 women between the ages of 50 and 54 yr, BUA had a sensitivity of 68% and a specificity of 67% for low BMD at the hip or spine (44). Likewise in a study of 1000 women between the ages of 45 and 49 yr, BUA was not successful in predicting women with low DXA measurements (45). Only 44% of the women whose spinal DXA measurement was in the lowest quartile were in the lowest BUA quartile (45). The same conclusion was reached when QUS and DXA measurements were obtained in 1000 women with a broader age range, between 20 and 93 yr (46). Defining osteoporosis as a T-score of < -2 at the spine, QUS had a sensitivity of 83% and a specificity of 57%. For the femoral neck, the sensitivity was 86% while the

Table 2
Sensitivity and Specificity of QUS at Various Cutpoints
for the Diagnosis of Osteoporosis Defined as a T-Score Less
Than -0.25 at the Spine, Total Hip, and/or Femoral Neck

	<i>Sensitivity</i>	<i>Specificity</i>
QUS T-score < -2	24%	93%
QUS T-score < -1	62%	72%
QUS T-score < 0	89%	32%

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specificity was 64%. It was concluded that no site can predict the status of another site with sufficiently high accuracy to be clinically useful (46). A similar conclusion was reached using QUS evaluation in a community setting (47).

In contrast, an evaluation of five different calcaneal QUS instruments has noted that the sensitivity and specificity of all the devices for the diagnosis of osteoporosis was approx 80% (48). It was concluded that the instruments provide a reasonable alternative to DXA for the initial evaluation of patients at risk for osteoporosis. Likewise, QUS of the phalanges has been reported to be effective for the screening of osteoporosis in postmenopausal women (49). Interestingly, this conclusion was reached with a sensitivity of 100% but a specificity of 40–45% (49).

In the evaluation of the individual patient it is important not only to diagnose osteoporosis, but to correctly identify those individuals who do not have the disease. If one examines perimenopausal women age 45–55 yr, at a sensitivity of 100% (all patients diagnosed who have the disease) the specificity of QUS is 31% (50). Thus to insure that no patients who actually have the disease go undiagnosed, 69% of individuals who do not have the disease are included (false positives). This discriminatory ability is not improved when evaluating women representing a broader age range, 50–85 yr (Table 2) (37). Thus, because of the differences in bone loss at different skeletal sites, the benefits of increased ease of osteoporosis diagnosis with ultrasound instruments will need to be balanced against potentially unnecessary treatment in some normal patients. If a lower sensitivity is accepted, fewer normal patients will be included but some osteoporotic patients will go undiagnosed (37,50). Measurement of bone mass with peripheral instruments may prove to be a valuable additional risk factor in evaluating women: a low value is indicative of osteoporosis (Table 2), but a high value does not indicate the absence of the disease in the spine and/or hip.

QUANTITATIVE ULTRASOUND AS AN INDICATOR OF FRACTURE RISK

Prospective studies have demonstrated the utility of QUS in the evaluation of older women at risk of hip fracture (51–54). The relative risk of hip fracture for every standard deviation decrease in the ultrasound measurements is similar to the risk for every standard deviation decrease in femoral neck BMD (Table 3) (52–54). After controlling for femoral neck BMD, the ultrasound measurements remained as significant predictors of fracture. These observations have been suggested as evidence that QUS measures other properties of bone in addition to density. Another explanation is that both QUS and BMD

Table 3
Relative Risk of Hip Fracture for Every
Standard Deviation Decrease in Femoral
Neck BMD or QUS Measurements

	<i>Relative risk</i>
Femoral neck BMD	1.9 (1.6–2.4)
BUA	2.0 (1.6–2.4)
SOS	1.7 (1.4–2.1)

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principally measure bone density; however, QUS and BMD have different sources of error and therefore contribute independently to fracture risk (53).

Retrospective studies have also demonstrated the ability of QUS to discriminate patients with hip fractures (55–66). As with the prospective studies, these reports indicate that QUS is able to identify patients with hip fractures as well as DXA and does so independently of BMD. Furthermore, combining the results from multiple QUS measurement sites appears to improve hip fracture discrimination, with a sensitivity and specificity of 94% (63). Thus, despite the relatively poor correlation between QUS values and DXA of the femoral neck, low QUS values appear to be comparable to low BMD values as predictors of hip fracture risk in older women. QUS values are also able to differentiate women with and without vertebral fractures (67–72). For every standard deviation decrease in QUS values the risk of spine fracture increases 1.3–2.6 fold. This is similar to the increase in risk for every standard deviation decrease in lumbar spine BMD (68). A prospective study has documented the observational results, for every standard deviation decrease in BUA the risk of vertebral fracture increases 1.5-fold (73).

QUS also predicts non-vertebral fracture risk in early postmenopausal women (71,74) and in young women performing strenuous physical activities (75). In a prospective study, the combination of QUS with DXA appears to improve non-spine fracture discrimination (76). In a cross-sectional analysis, QUS predicted non-vertebral fractures, but the combination of QUS and DXA did not improve fracture discrimination (77). In contrast to the improvement in hip fracture discrimination with QUS measurement at multiple sites (63), QUS assessment at multiple skeletal sites does seem to improve non-vertebral fracture risk assessment (78). A recent study demonstrates that QUS measurements are also able to discriminate between men with and without fractures (79). Thus, the combined studies (52–79) indicate that QUS measurements in individuals at risk of fracture are useful indicators of skeletal fragility.

QUANTITATIVE ULTRASOUND UTILITY TO MONITOR CHANGES IN BONE MASS

QUS Changes at the Menopause

Bone loss at the menopause appears to predominantly affect trabecular sites because of the greater surface area of bone at those locations. Hence, QUS of the calcaneus (a predominantly trabecular site) is a potentially valuable tool to detect perimenopausal bone loss. The QUS measurements have been reported to be lower, but not significantly, in early postmenopausal women (50). In contrast, another report suggests that BUA, but not SOS, is

significantly decreased in early postmenopausal women (80). The most likely explanation for these apparent differences is that the magnitude of the annual changes in the QUS measurements is not great. This limits their use as a follow-up tool in individuals (81).

QUS to Detect Skeletal Changes in Response to Therapy

QUS has been utilized to assess skeletal changes in response to hormone replacement therapy (82–87), calcitonin (88), and combinations of therapies including bisphosphonates (89).

In some studies, hormone replacement therapy has been shown to prevent the decrease in QUS values subsequent to menopause (82–84). In contrast, other studies suggest that while hormone replacement therapy maintains BMD it does not prevent the decrease in QUS values (85,86). A potential explanation for these divergent results is that QUS of the heel in postmenopausal women taking hormone replacement therapy is affected by variation in vitamin D receptor and estrogen receptor loci, jointly (87). A two-locus genotype present in 9.5% of women was responsible for over 30% of the total hormone replacement therapy-related heel ultrasound difference in the whole study group (87). These findings could explain the different results, but if correct would suggest that QUS of the heel can not be routinely used to monitor the response to hormone replacement therapy.

In a prospective study of 112 women, salmon calcitonin nasal spray treatment for 2 yr prevented the decline in lumbar spine BMD and in QUS measurements at the heel (88). In contrast, a report evaluating the response of 673 women to either estrogen, bisphosphonates, high dose vitamin D plus calcium, or combinations thereof concluded that changes in calcaneal QUS values are not a reflection of changes in axial BMD and are not a substitute for direct DXA measurement of the spine and hip of treated patients (89). Thus, at present the combined reports (82–89) would suggest that QUS cannot be routinely used to monitor the response to therapy.

QUANTITATIVE ULTRASOUND USE IN CLINICAL SITUATIONS THAT MAY AFFECT BONE METABOLISM

QUS Use in Children

A prospective study in children and adolescents has demonstrated that QUS values change in a manner similar to DXA in this age group (90). QUS values are able to differentiate normal and osteopenic children (91), and those children with chronic rheumatic diseases (92).

QUS Use to Evaluate Weight-Bearing Activities

Weight bearing activities have been suggested as a means of increasing bone density. Brisk walking (93) and professions (94) and sports (95) that require weight bearing have been reported to increase heel QUS values. This suggests that measurement of calcaneal QUS values may reflect the positive effects of weight-bearing activities on the skeleton.

QUS Values During Pregnancy

There are very few studies evaluating the effect of pregnancy on the maternal skeleton because prior to the introduction of QUS the techniques involved exposure to radiation. Longitudinal studies have demonstrated that during pregnancy the calcium requirements of the fetus cause a significant decrease in QUS values at the maternal heel (96,97). The detrimental effects of pregnancy on the maternal skeleton appear to be greater in adoles-

cents (97). These studies suggest that the combination of fetal skeletal demands and those of a growing adolescent skeleton place significant demands on the maternal skeleton.

QUS Values in Patients with Anorexia Nervosa

BUA of the calcaneus has been shown to be decreased in patients with anorexia nervosa (98). BUA correlates significantly with spine and femoral neck BMD, body mass index, and duration of the disease in these patients (98).

QUS Values in Patients on Dialysis

It is well recognized that renal disease has a negative effect on bone mass. QUS values at the phalanges are significantly lower in men and women with end-stage renal disease than in age-matched controls (99,100). In addition, in these patients the QUS values strongly correlate with bone alkaline phosphatase, suggesting that QUS may be a useful tool in the evaluation of individuals on maintenance dialysis (100).

QUS Values in Patients on Glucocorticoids

Bone loss is common in patients requiring glucocorticoid treatment. QUS values at the calcaneus are significantly lower in women with inflammatory bowel disease taking corticosteroids than in patients not requiring corticosteroids and in controls (101).

QUS Values in Hyperparathyroidism

Primary hyperparathyroidism is thought to preferentially affect cortical bone sites. Although QUS values at the calcaneus are decreased in patients with hyperparathyroidism, these values are decreased to a lesser extent than BMD (102). Surgical treatment of hyperparathyroidism is associated with increases in both QUS and BMD values (103).

QUS Values in Paget's Disease

QUS at the tibia has been evaluated in 10 patients with Paget's disease affecting a single tibia (104). As expected, BMD of the affected tibia is increased. However, the QUS value is significantly decreased in the involved tibia. This strongly suggests that QUS detects important changes in bone quality in Paget's disease of bone, which are unrelated to calcium content (104).

QUS Values in Institutionalized Individuals

Osteoporosis is a significant problem in institutionalized individuals. QUS values at the calcaneus continue to decline in elderly institutionalized elderly women and are able to differentiate women with previous non-vertebral fractures (105). QUS values are also decreased significantly in institutionalized adults with mental retardation (106). Given the difficulties of quantitating bone mass in individuals who do not have easy access to DXA instruments, the portability of QUS may make it an ideal technique to assess skeletal status in these patients.

FUTURE DIRECTIONS IN THE USE OF ULTRASOUND CLINICALLY

It is apparent that QUS measurements are capable of predicting osteoporotic fracture risk (51–79). In contrast, in the individual patient, QUS values appear to lack the sensitivity and specificity required to enable the use of QUS as the sole diagnostic technique

upon which to base therapeutic decisions (37,43–50). Part of this discrepancy may relate to the criteria used to diagnose osteoporosis. The World Health Organization criteria for diagnosis may not be applicable to calcaneal QUS measurements (107,108). At a T-score ≤ -2.5 , the prevalence of osteoporosis in healthy postmenopausal women is 17, 16, and 12% for lumbar spine, femoral neck, and total hip BMD, respectively. When the same definition is used for calcaneal QUS measurements, the prevalence ranges from 2 to 8%, depending on the device. The corresponding T-score threshold for the QUS devices resulting in similar prevalence to the BMD measurement is -1.8 (108). However, even at this QUS cutpoint a significant percentage of women with osteoporosis at the spine, femoral neck, and/or hip go undiagnosed (Table 2) (37).

Future research will need to focus on the outcomes in those women who have discrepant QUS and BMD values. Pending those results, it is clear that women with low QUS and BMD measurements are at greatest fracture risk.

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8

Molecular Markers of Bone Turnover

Basic and Analytical Aspects

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and Markus J. Seibel, MD*

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SUMMARY

The development of new markers of bone metabolism has greatly enriched the spectrum of serum and urine analytes used in the assessment of skeletal pathologies. Both markers of bone formation and of bone resorption are today widely used in experimental and clinical situations to assess bone turnover, rates of bone loss, future fracture risk and therapeutic efficacy. It should be borne in mind, however, that many of the compounds used as markers of bone turnover may reflect, at least to a certain degree, both bone formation and bone resorption. Furthermore, most of these markers are present in tissues other than bone and may therefore be influenced by nonskeletal processes as well. Thirdly, changes in biochemical markers of bone turnover are usually not disease specific, but reflect overall alterations in skeletal metabolism independent from the underlying cause. Therefore, results of bone marker measurements should always be interpreted against the background of their basic science.

INTRODUCTION

Bone mass is the net result of two counteracting metabolic processes, i.e., bone formation and bone resorption. Under normal conditions, bone formation and bone resorption are coupled to each other (1,2). However, somatic growth, normal aging, metabolic bone diseases, states of increased or decreased mobility, therapeutic interventions and other conditions are characterized by more or less pronounced imbalances in bone turnover. The long term result of such imbalances in bone turnover are often changes in bone mass and structure, parameters which are usually assessed by radiological and densito-

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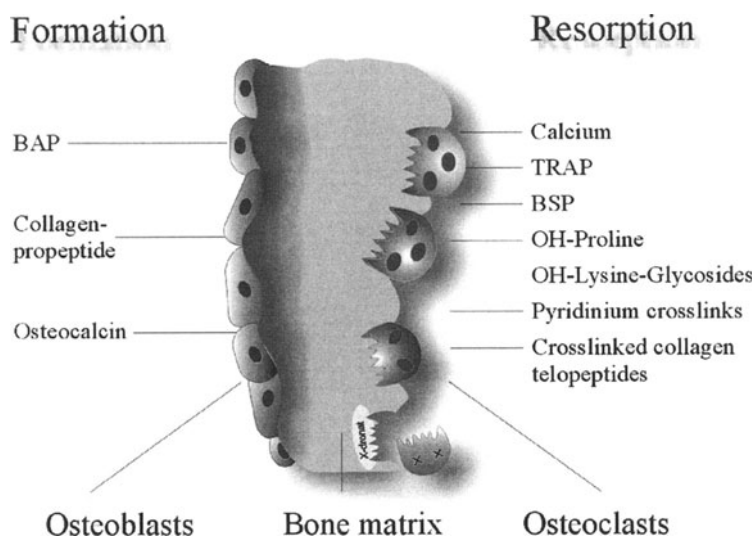


Fig. 1. Schematic display of biochemical markers of bone turnover being synthesized through bone formation (left side) or released by matrix degradation via osteoclasts (right side).

metric techniques. In contrast to these more static measures, molecular markers of bone metabolism are helpful tools to detect the dynamics of the metabolic imbalance itself.

In recent years, the isolation and characterization of cellular and extracellular components of the skeletal matrix have resulted in the development of biochemical markers that specifically reflect either bone formation or bone resorption. These new biochemical indices have greatly enriched the spectrum of analytes used in the assessment of skeletal pathologies. Although the various serum and urinary markers of bone turnover include enzymes, nonenzymatic peptides and mineral components, they are usually classified according to the metabolic process they are considered to reflect. For clinical purposes, therefore, markers of bone formation are distinguished from indices of bone resorption (Fig.1; Tables 1 and 2). From a biochemical perspective, however, bone consists of a calcified organic matrix. The latter is composed of 90% collagen (type I) and 10% of noncollagenous proteins, such as osteocalcin or bone sialoprotein. Therefore, the various markers may also be classified according to the biological compartment they belong to, i.e., parameters of osteoblast and osteoclast enzyme activity, components released during the formation and breakdown of the organic bone matrix, and parameters of the inorganic skeletal matrix, namely calcium and phosphorus.

MARKERS OF BONE FORMATION

Bone formation markers are direct or indirect products of active osteoblasts expressed during different phases of osteoblast development and reflecting different aspects of osteoblast function and bone formation. All markers of bone formation are measured in serum or plasma.

Alkaline Phosphatase

Alkaline phosphatase (AP) is a ubiquitous, membrane-bound tetrameric enzyme attached to glycosyl-phosphatidylinositol moieties located on the outer cell surface (3).

Table 1
Markers of Bone Formation

<i>Marker (abbreviation)</i>	<i>Tissue of origin</i>	<i>Analytical specimen</i>	<i>Analytical method</i>	<i>Specificity</i>
Alkaline phosphatase (AP, TAP)	Bone, liver, intestine, kidney, placenta	Serum	Colorimetric	Specific for bone formation only in the absence of liver or biliary disease
Bone-specific alkaline phosphatase (BAP)	Bone	Serum	Colorimetric, electrophoretic, precipitation, IRMA, EIA	Specific product of osteoblasts. Some assays show up to 20% cross-reactivity with liver isoenzyme (LAP)
Osteocalcin (OC, BGP)	Bone, platelets	Serum	RIA, IRMA, ELISA	Specific product of osteoblasts; many immunoreactive forms in blood; some may be derived from bone resorption
Carboxy terminal propeptide of type I procollagen (PICP)	Bone, soft tissue, skin	Serum	RIA, ELISA	Specific product of proliferating osteoblasts and fibroblasts
Amino-terminal propeptide of type I procollagen (PINP)	Bone, soft tissue, skin	Serum	RIA, ELISA	Specific product of proliferating osteoblast and fibroblasts; partly incorporated into bone extracellular matrix

The precise function of the enzyme is yet unknown (4), but it obviously plays an important role in osteoid formation and mineralization. The total AP serum pool (TAP) consists of several dimeric isoforms, which originate from various tissues: liver, bone, intestine, spleen, kidney, and placenta. In addition, certain tumors may express macromolecular forms of AP (e.g., “Nagao AP”) (5,6).

The physiological isoforms of AP are coded by four gene loci, including three tissue-specific and one nontissue-specific gene on chromosome 1. The latter encodes for the most abundant isoforms, namely bone, liver, and kidney AP. The differences between these nonspecifically encoded isoenzymes are solely due to post-translational modifications in the carbohydrate moiety (7). In adults with normal liver function, approx 50% of the total AP activity in serum is derived from the liver, whereas 50% arises from bone (8). In children and adolescents the bone-specific isoenzyme predominates (up to 90%) because of skeletal growth (9). The bone isoforms can be separated by HPLC into B/I, B1, and B2, with B2 contributing to 35% of serum total AP (10).

Many techniques have been developed to differentiate between the two main isoforms of circulating AP, including heat denaturation, electrophoresis, precipitation, selective inhibition, and, more recently, immunoassays. All of these methods make use of the different physicochemical and molecular properties of the two isoenzymes (11–15). In healthy adults, most methods show a good correlation between bone specific and total

Table 2
Markers of Bone Resorption

<i>Marker (abbreviation)</i>	<i>Tissue of origin</i>	<i>Analytical specimen</i>	<i>Analytical method</i>	<i>Specificity</i>
Hydroxyproline, total and dialyzable (OH-Pro, OHP)	Bone, cartilage, soft tissue, skin	Urine	Colorimetric HPLC	All fibrillar collagens and partly collagenous proteins, including C1q and elastin; present in newly synthesized and mature collagen
Pyridinoline (PYD; Pyr)	Bone, cartilage, tendon, blood vessels	Urine	HPLC ELISA	Collagens, with highest concentrations in cartilage and bone; absent from skin; present in mature collagen only
Deoxypyridinoline (DPD, d-Pyr)	Bone, dentin	Urine	HPLC ELISA	Collagens, with highest concentration in bone; absent from cartilage or skin; present in mature collagen only
Carboxy terminal crosslinked telopeptide of type I collagen (ICTP)	Bone, skin	Serum	RIA	Collagen type I, with highest contribution probably from bone; may be derived from newly synthesized collagen
Carboxy terminal crosslinked telopeptide of type I collagen (α -CTX, β -CTX)	All tissues containing type I collagen	Urine Serum	ELISA RIA	Collagen type I, with highest contribution probably from bone
Amino-terminal crosslinked telopeptide of type I collagen (NTx)	All tissues containing type I collagen	Urine (α -/ β) Serum (β only)	ELISA RIA	Collagen type I, with highest contribution probably from bone
Hydroxylysine glycosides	Bone, soft tissue, skin, serum complement	Urine	HPLC	Collagens and collagenous proteins; glycosyl-galactosyl-OHLys in high proportion in collagens of soft tissues, and C1q; Galactosyl-OHLys in high proportion in skeletal collagens
Bone sialoprotein (BSP)	Bone, dentin, hypertrophic cartilage, carcinomas	Serum	RIA ELISA	Synthesized by active osteoblasts and laid down in bone extracellular matrix. Appears to reflect osteoclast activity
Tartrate-resistant acid phosphatase (TRAP)	Bone blood	Plasma serum	Colorimetric RIA	Osteoclasts, platelets, erythrocytes

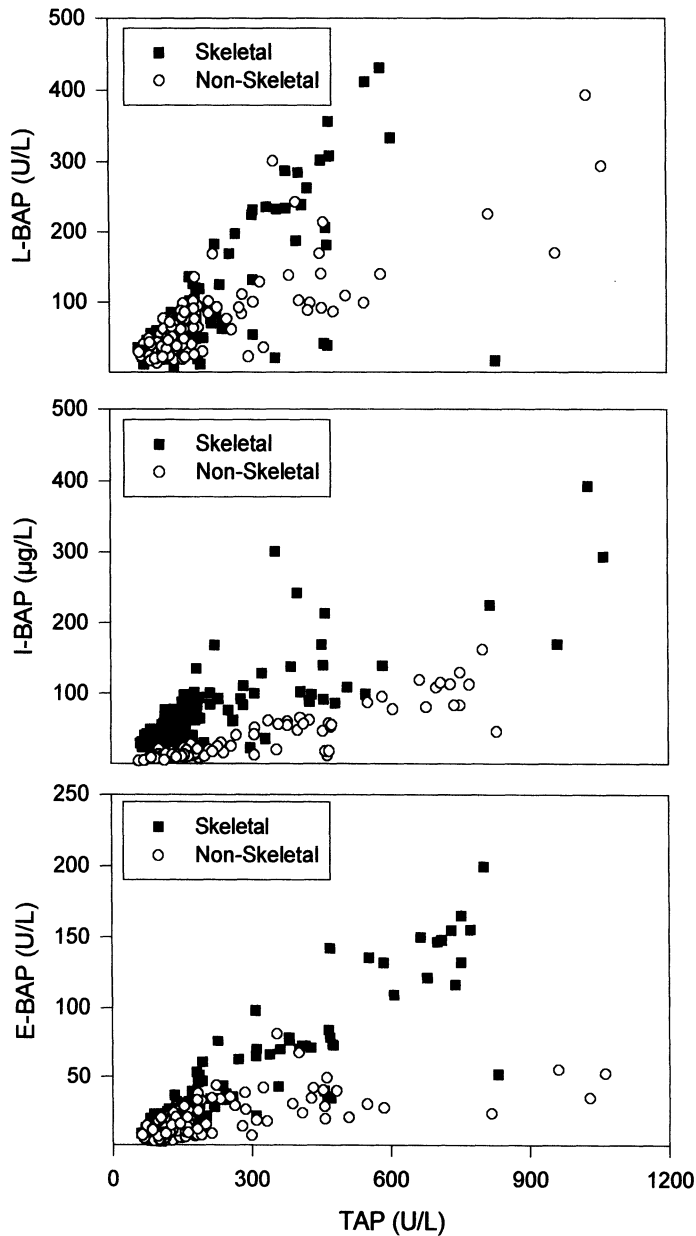


Fig. 2. Correlations between serum TAP and the various serum BAP measurements. Patients with nonskeletal disease had chronic hepatic failure, chronic obstructive pulmonary disease, or chronic renal failure. Patients with skeletal disease had Paget disease of bone, PHPT, SHPT, or metastatic bone disease (17).

AP. The novel immunoassays allow simple and rapid quantitation of either enzyme activity or enzyme mass. However, like all of the other techniques, even these assays show a certain degree of crossreactivity between bone and liver AP (15–20%). Therefore, in subjects with high liver AP, results of bone AP measurements may be artificially high, leading to false positive results (7,16,17; Fig. 2).

Serum total AP is the most widely used marker of bone metabolism due to the wide availability of inexpensive and simple methods. Once liver disease is ruled out, serum levels of total AP provide a good impression of the extent of new bone formation and osteoblast activity (18,19). From a clinical perspective, however, detection of the bone-specific AP (BAP) isoenzyme is increasingly preferred because of its higher specificity (8,20,21).

Osteocalcin

Osteocalcin (OC) is a 5.8 kDa, hydroxyapatite-binding, protein exclusively synthesized by osteoblast, odontoblasts and hypertrophic chondrocytes (22–24). One of the major characteristics of OC are three vitamin-K dependent, gamma-carboxyglutamic acid (Gla) residues, which are responsible for the calcium binding properties of the protein (25). At its carboxy-terminus, OC can also interact with other proteins, including cell surface receptors. OC is therefore active in the organization of the extracellular matrix. Earlier research has suggested that OC is involved in the process of osteoid mineralization, as the protein is expressed mainly during this phase of bone formation. However, although OC has been studied for more than 20 yr, its precise function has yet to be determined. More recently, new light has been cast on this issue through the development of an osteocalcin knockout mouse model. Unexpectedly, these animals have increased cortical and trabecular thickness, and their bones seem mechanically more stable than those of the wild type (26). Although the knockout model awaits further characterization, it seems that OC is involved in the bone remodeling process and may act via a negative feed back mechanism.

OC is considered as a specific marker of osteoblast function (27). It is estimated that, directly after its release from osteoblasts, the largest part of the newly synthesized protein is incorporated into the extracellular bone matrix where it constitutes approx 15% of the noncollagenous protein fraction. A smaller fraction is released into the circulation where it can be detected by immunoassays (28–34). Serum levels of immunoreactive OC have been shown to correlate well with the bone formation rate as assessed by histomorphometry (35). However, the peptide is readily subject to rapid degradation in serum, so that both intact peptides and OC fragments of various sizes coexist in the circulation (36–38). Furthermore, since OC is incorporated into the bone matrix, some investigators have suggested that OC fragments may be released even during bone resorption. This may be particularly true for some smaller N-terminal fragments of OC, which are found in individuals with high bone turnover (39–41). The ensuing heterogeneity of OC fragments in serum results in considerable limitations in the clinical application of this *a priori* highly specific marker. Thus, the various assays used to measure OC in serum detect fragments of various sizes and usually, epitope specificity and antibody cross-reactivity of the assays are ill defined. In practice, different immunoassays have routinely yielded such varying results in measurements that they are incomparable (42–44).

Two-site immunoassays, utilizing antibodies detecting different parts of the OC molecule, have been introduced that detect the intact 1–49 OC molecule. However, only one-third of the total OC serum pool represents intact OC, and due to the instability of OC in serum, rapid loss of immunoreactivity is seen with these assays when samples are stored for more than 1 h at room temperature. To circumvent this problem, newer assays measure the largest degradation product of OC, the 1–43 (N-terminal/mid-molecule) fragment. This fragment, which represents 1/3 of the circulating OC pool, is a result of

proteolytic degradation of the intact molecule and may in part be generated by active osteoblasts. Although little is known about the function of the N-terminal fragment, its measurement eliminates in part the problem of pre-analytical instability (44,45). However, quick processing of the blood sample after drawing is essential for most assays since a loss of reactivity is noted within a few hours at room temperature. The same applies to sera subjected to multiple thawing, or prolonged storage at temperatures above -25°C .

Procollagen Type I Propeptides

Procollagen type I propeptides are derived from collagen type I, which is the most commonly occurring collagen of bone (46). However, type I collagen is also found in other tissues such as skin, dentin, cornea, vessels, fibrocartilage, and tendons. In bone, collagen is synthesized by osteoblasts in the form of pre-procollagen. These precursor molecules are characterized by short terminal extension-peptides: the amino (N-) terminal propeptide (PINP) and the carboxy (C-) terminal propeptide (PICP) (47). After secretion into the extracellular space, the globular trimeric propeptides are enzymatically cleaved (48) and liberated into the circulation. PICP has a Mw of 115 kDa, is stabilized by disulfide bonds, cleared by liver endothelial cells via the mannose receptor and therefore has a short serum half-life of 6–8 min (49,50). PINP has a Mw of only 70 kDa, is rich in proline and hydroxyproline and is eliminated from the circulation by liver endothelial cells by the scavenger receptor. For a current review of procollagen propeptides, *see* ref. 70. Since both PICP and PINP are generated from newly synthesized collagen in a stoichiometric fashion, the propeptides are considered quantitative measures of newly formed type I collagen. Although type I collagen propeptides may also arise from other sources, most of the nonskeletal tissues exhibit a slower turnover than bone, and contribute very little to the circulating propeptide pool.

Both propeptides are currently measured by specific, polyclonal based immunoassays (51,52). Different studies have shown good correlations between serum PICP levels and the rate of bone formation or serum TAP activity (53,54). While the clinical relevance of PICP in the evaluation of metabolic bone diseases is still viewed with skepticism (55,56), serum PINP appears to be of greater diagnostic validity. From a practical point of view, the thermostability of the propeptides is an advantage in that extended transport and frozen storage times can be well tolerated without significant loss. The propeptides share these properties with most of the parameters of collagen metabolism (e.g., crosslinks, ICTP, NTx, CTx, hydroxyproline; *vide infra*).

MARKERS OF BONE RESORPTION

Except for tartrate-resistant acid phosphatase (*vide infra*), the majority of bone resorption markers are degradation products of bone collagen (Fig. 3). Only recently, noncollagenous proteins such as bone sialoprotein or osteopontin have been investigated as markers of bone turnover. Furthermore, most assays for bone resorption markers were confined to urine, but newer assays are now available also for serum measurements.

Hydroxyproline

Hydroxyproline (OHP) is formed intracellularly from the post-translational hydroxylation of proline and constitutes 12–14% of the total amino acid content of mature collagen. 90% of the OHP liberated during the degradation of bone collagen is primarily

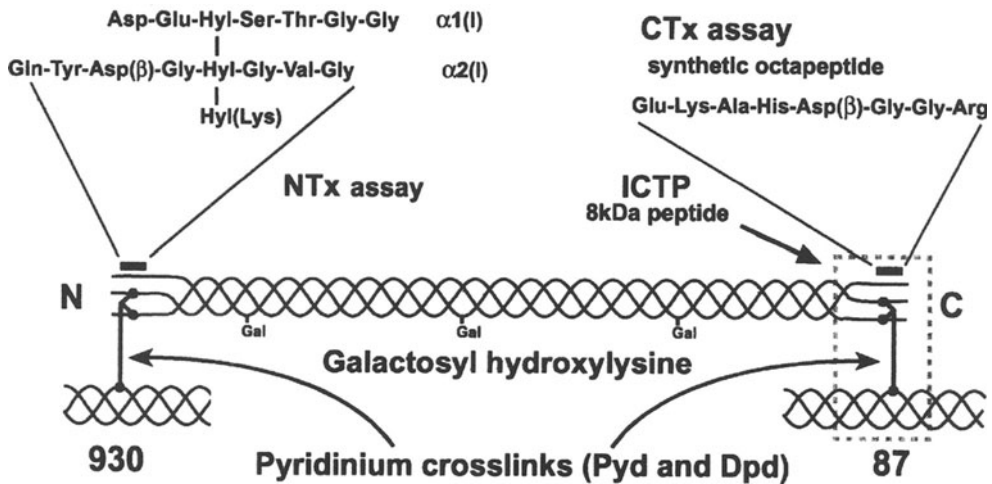


Fig. 3. The collagen type I triple-helix and the molecular origin of markers of bone turnover: PYD- and DPD-crosslinks, N- and C-terminal telopeptides, and OH-lysine glycosides.

metabolized in the liver (57). Subsequently, it is excreted in the urine where it may be detected either as free or peptide-bound hydroxyproline by colorimetric or HPLC methods (58,59). Urinary OHP is usually considered an index of bone resorption. However, it should be noted that significant amounts of urinary OHP are derived from the degradation of newly synthesized collagens (60). In addition, hydroxyproline can be found in other tissues such as the skin (61) and, moreover, liberated from the metabolism of elastin and C1q (62). Urinary hydroxyproline is therefore considered an unspecific index of collagen turnover and, consequently, has been largely replaced by more specific techniques.

Hydroxylysine-Glycosides

These collagen-specific amino acids are formed during the posttranslational phase of collagen synthesis and occur in two forms, namely glycosyl-galactosyl-hydroxylysine (GGHL) and galactosyl-hydroxylysine (GHL) (63). Both components are released into the circulation during collagen degradation and may be measured in urine by HPLC after appropriate derivatization (64). The intrinsic advantage of hydroxylysine over that of hydroxyproline as a marker of bone resorption is that the glycosylated forms are not metabolized and are not influenced by dietary components (63,64). Moreover, GGHL is present in skin and C1q, while GHL is more specific for bone. Thus the ratio of GGHL/GHL may allow for the recognition of existent tissue specificity. Although the hydroxylysines have potential as markers of bone resorption (65–67), their major disadvantage is presently the absence of a convenient immunoassay format.

Hydroxypyridinium Crosslinks of Collagen

Hydroxypyridinium crosslinks of collagen are formed during the extracellular maturation of fibrillar collagens. The 3-hydroxy-pyridinium derivatives pyridoline (PYD) and deoxypyridoline (DPD) are trifunctional crosslinks, as such bridging several collagen peptides and mechanically stabilizing the collagen molecule (68–70). During bone resorption, crosslinked collagens are proteolytically broken down and the crosslink components are released into the circulation and the urine (71). In contrast to hydroxy-

proline, the measurement of hydroxypyridinium crosslinks is not influenced by the degradation of newly synthesized collagens. Their levels strictly reflect the degradation of mature, i.e. crosslinked collagens, and thus processes of collagen breakdown. This theoretically predicted specificity has been experimentally confirmed by close correlations between crosslink excretion and histomorphometric or dynamic parameters of bone resorption (72,73). In addition, the urinary excretion of pyridinium crosslinks is independent of dietary sources since neither PYD nor DPD are taken up from food (74). Finally, the two hydroxypyridinium components show a high specificity for skeletal tissues. While PYD is found in cartilage, bone, ligaments and vessels, DPD is almost exclusively found in bone and dentin. Neither derivative is present in the collagen of skin or in other sources such as C1q or elastin (62,75,76). Since bone has a much higher turnover than cartilage, ligaments, vessels or tendons, the amounts of PYD and DPD in serum or urine are mainly derived from the skeleton. Thus, the pyridinium crosslinks are currently viewed the best indices for assessing bone resorption (77–79).

Initially, PYD and DPD were quantified in urine by reverse-phase ion-paired HPLC technique, combined with a prefractionation step using cellulose partition chromatography, and hydrolysis of urine samples to convert all crosslinks into the peptide free forms (76,80). More recently, automated techniques for the measurement in serum have been described (81,82). Analysis of urine by HPLC without initial hydrolysis showed that 40–50% of the crosslinks were present in peptide-free form (83). Although the amounts of free and peptide-bound crosslinks seem to vary with bone pathologies, today direct immunoassays for *free* and peptide-bound crosslinks are widely used. Under normal conditions, these assays have been shown to produce results similar to those provided by the traditional HPLC technique (84–86; Fig. 4).

Crosslinked Teloptides of Type I Collagen

As crosslinking of collagen always involves a specific molecular region, namely the aminoterminal (NTP) and the carboxyterminal (CTP) telopeptide, assay developments aimed for the detection of these components in urine and serum. The first peptide assay ever was a RIA for the carboxyterminal type I collagen telopeptide (ICTP) in serum (87). The respective antibodies were raised against a crosslink-containing collagen peptide (mol wt = 8.5 kDa) isolated from human bone. The antigenic determinant requires a trivalent crosslink, including two phenylalanine-rich domains of the telopeptide region of the α -1 chain of type I collagen. Divalently and noncrosslinked peptides do not react with the antibody, nor do peptides isolated from skin. Despite the fact that the initial peptide contained pyridoline, the assay also detected other crosslink forms such as deoxypyridoline or pyrrole crosslinks. The ICTP assay appears to be sensitive for pathological bone resorption as seen in multiple myeloma, metastatic bone disease and other degradation processes involving rapid breakdown of skeletal and non-skeletal type I collagen (88).

Another set of immunoassays involve the carboxyterminal telopeptide of type I collagen, abbreviated CTX (89). Employing a polyclonal antiserum against a synthetic octapeptide containing the crosslinking site, a first ELISA (termed β -CTX) recognized the C-terminal type I collagen telopeptide containing an isoaspartyl (= β -aspartyl) peptide bond in its L-enantiomeric form (90). The β -L-aspartyl is considered to result mainly from the aging of extracellular proteins. Only one peptide strand is necessary for immunoreactivity. Meanwhile, a monoclonal-based RIA for the nonisomerized octapeptide (EKAH- α D-GGR) in urine has been developed (“ α -CTX”) (91). Simultaneous mea-

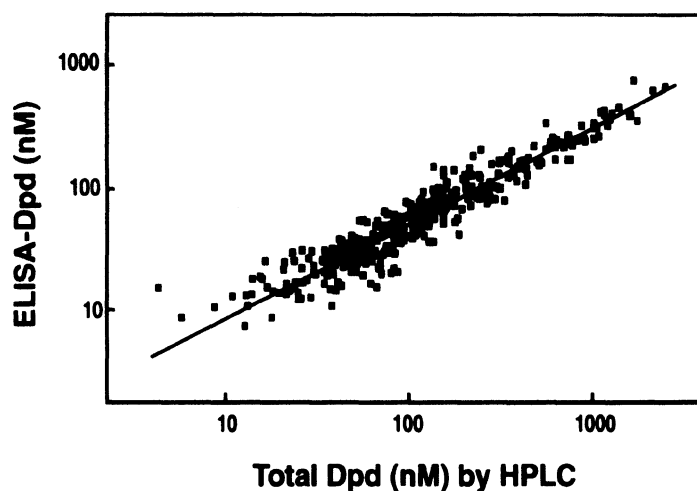


Fig. 4. Relationship between urinary concentrations of free DPD measured by ELISA and total DPD assayed by HPLC. The data, normalized by logarithmic transformation, showed a high correlation ($r = 0.95$) between the two methods with a regression equation of $\log_e y = 0.76 \pm 0.01 \log_e x + 0.42 \pm 0.06$, giving the relationship $\text{ELISA} = 1.52(\text{total})^{0.76}$ between values for the two assay methods (85).

surement of both forms may be used to calculate the ratio of α -CTX/ β -CTX as an index of bone turnover. This ratio has been shown to be elevated in the urine of patients with untreated Paget's disease of bone, where rapid bone formation and resorption results in an increase of α -CTx (nonisomerized octapeptide) (92).

Further to isomerization, many proteins are also subjected to racemization of certain residues. Both processes are considered an effect of aging, as the extent of racemization and isomerization increases with the time elapsed since synthesis of the protein. Additionally, antibodies directed against the D-aspartic acid residues in the CTX molecule have been described (93). Thus, immunoassays now exist for all four possible isomers of the CTX molecule: the native α -L form, the β -isomerized isoaspartyl peptide (β L), and the respective racemized forms α -D and β -D. The differential use of these assays may possibly provide information on the age-dependent changes of collagen in health and disease (94), although the clinical relevance is questionable.

Recently, a sandwich ELISA for the measurement of β -CTX in serum has been developed. When this assay was described initially, it utilized a polyclonal antiserum against collagenase-digested collagen type I together with the β -isomerized C-terminal octapeptide attached to the ELISA plate (95). The newer assay uses two monoclonal antibodies, which purportedly recognize only dipeptides containing a crosslink and two β -isomerized peptides with the same sequence as shown for the urinary assay. Although there is no direct evidence that the antigenic determinant in serum must contain a crosslink for immunoreactivity, this is indeed highly probable since β -transformation occurs slower than crosslinking, and single peptides do not react in the serum CTX-ELISA. However, the crosslink component may be any kind of crosslink, similar to the ICTP antigen in serum. Presently, standards for the original serum CTX-ELISA are made from antigens immunopurified from human urine, but automated assays (Boehringer-Mannheim) utilize synthetic antigens. Serum and urinary CTX values are highly correlated ($r = 0.86$) suggesting that the antigen is similar in both analytical media.

Another peptide assay measures the crosslinked N-terminal telopeptide of type I collagen. This assay (termed NTX-assay) utilizes a monoclonal antibody (1H11) raised against an epitope on the α -2 chain of type I collagen (96). However, the antibody seems to react with several crosslinking components, and the presence of a pyridinium crosslink is not essential for reactivity. As a matter of fact, digests of skin collagen exhibited similar reactivity with the NTX assay as skeletal extracts (97). Both the monoclonal antibody and the assay format are identical for the urine and the serum based assays. Expectedly, both assays show good correlation, and the analyte seems to be stable at room temperature and during up to three freeze-thaw cycles (98).

Bone Sialoprotein (BSP)

BSP is a phosphorylated glycoprotein with an apparent Mw of 70–80 kDa, which accounts for 5–10% of the noncollagenous matrix of bone (99,100). The protein has been shown to be a major synthetic product of active osteoblasts and odontoblasts, but was also found in osteoclast-like and malignant cell lines. Consequently, BSP or its mRNA is detected mainly in mineralized tissue such as bone, dentin and at the interface of calcifying cartilage (101–103). Intact BSP contains an Arg-Gly-Asp (RGD) integrin recognition sequence, improves the attachment of osteoblasts and osteoclasts to plastic surfaces (104), binds preferentially to the α 2 chain of collagen, nucleates hydroxyapatite crystal formation in vitro (105) and appears to enhance osteoclast mediated bone resorption (104). The protein is therefore considered to play an important role in cell-matrix-adhesion processes and in the supramolecular organization of the extracellular matrix of mineralized tissues.

More recently, immunoassay techniques have been developed that measure immunoreactive bone sialoprotein in serum (106,107). All of these assays are based upon polyclonal antisera, and little is known about the specific nature of the respective epitopes. Antibodies do not cross-react with noncollagenous proteins such as osteonectin, fibronectin or osteocalcin (107). In serum, the majority of BSP is bound to Factor H, a major regulator of the alternate complement pathway. Although this phenomenon is of unknown physiological relevance, BSP/Factor H binding studies suggest that current immunoassays do detect only a fraction of bioavailable BSP in serum (108). Based upon clinical data and the rapid reduction of serum BSP levels following intravenous bisphosphonate treatment, it is assumed that serum BSP reflects processes mainly related to bone resorption (109).

Tartrate-Resistant Acid Phosphatase (TRAP)

TRAP belongs to the family of ubiquitously occurring acid phosphatases, of which at least five different isoforms are known. These isoforms are expressed by different tissues and cells such as prostate, bone, spleen, platelets, erythrocytes, and macrophages. All acid phosphatases are inhibited by L(+)-tartrate, except band 5, which was therefore termed tartrate-resistant acid phosphatase (TRAP). Of the latter, 2 subforms, 5a and 5b are known, and recent research has shown that TRAP-5b is characteristic for osteoclasts (110). The origin of TRAP-5a is unknown, but may be expressed by macrophages. The two isoforms 5a and 5b are different in that 5a contains sialic acid, whereas 5b does not. So far, most assays for the measurement of TRAP in blood were colorimetric and detected both isoforms without differentiating between bands 5a and 5b. Only recently, specific immunoassays for TRAP 5b have been described and clinical results indicate that this marker may be useful to assess osteoclast activity (111,111a). The antibodies for these

assays were raised against material isolated from the spleen of a patient with hairy cell leukemia (112) or against TRAP 5b isolated from human cord plasma (113). For the conventional TRAP assays, care should be taken after phlebotomy to stabilize the enzyme since TRAP loses more than 20% of its activity per hour at room temperature. This can be prevented by the addition of citrate buffer to the sample (114).

PREANALYTICAL VARIABILITY OF BONE TURNOVER MARKER

Nonspecific variability in the measurement of chemical analytes is a major issue in clinical chemistry. Consideration and, where ever possible, tight control of factors not related to the specific process in question, is essential for the correct interpretation of laboratory results. At present, these caveats seem of particular relevance for the various molecular markers of bone turnover, as the degree of variability of some of these analytes has been shown to be rather substantial.

The term “analytical variability” mainly concerns issues of technical assay performance, i.e., measures of assay precision and accuracy (e.g., linearity, intra- and inter-assay variability, standardization). Recent studies showed that most assays lack the appropriate standardization to enable reliable comparison of results between different laboratories.

Ideally, biochemical markers of bone turnover, and the assays used for their quantitation, should be characterised by minimal and predictable variability and should be influenced as little as possible by preanalytical and analytical conditions. This, of course, is usually not the case. Therefore, changes in marker measurements, for example as a result of treatment interventions, should always be interpreted against the background of the respective marker’s total variability. As a rule, markers showing large changes in response to interventions also show substantial degrees of nonspecific variability. The more relevant preanalytical factors affecting marker variability are summarized in Table 3.

Technical Aspects of Preanalytical Variability

The choice of sample (i.e., serum vs urine), the mode of urine collection (i.e., 24-h collection vs first or second morning void), the appropriate preparation of the patient (i.e., minimizing the effect of diet or exercise before phlebotomy) and the correct processing and storage of specimens all influence the final analytical result (Table 3). Therefore, special care must be taken of these more technical issues, in particular, as they are modifiable and controllable.

While both alkaline phosphatases and procollagen propeptides have been shown to be rather stable at room temperature, serum osteocalcin and some other noncollagenous proteins are readily degraded even at temperatures as low as 4°C (115). Signal reduction is less pronounced when measuring smaller (already degraded) fragments of OC, e.g., OC(1–43) (44,45).

Both the free and conjugated forms of PYD and DPD have been shown to be stable in urine samples kept at room temperature for several weeks. Several reports show that pyridinium crosslinks can be stored at –20°C for years and that repeated freeze-thaw cycles of urine samples have no effect on the concentrations of PYD and DPD (116). Pyridinium crosslinks in aqueous solutions are unstable when subjected to intensive UV irradiation. Similar stability has been reported for the urinary N-terminal (NTX) and

Table 3
Preanalytical and Biological Characteristics of Bone Biomarkers

<i>Marker (abbreviation)</i>	<i>Recommended storage</i>	<i>Concentrations influenced by</i>	<i>Diurnal rhythms</i>
Total alkaline phosphatase (AP, TAP)	Stable < -20°C	Liver function	Negligible
Bone-specific alkaline phosphatase (BAP)	Stable < -20°C	Liver function	Negligible
Osteocalcin (OC, BGP)	Unstable > -80°C	Renal function	Significant
Carboxy terminal propeptide of type I procollagen (PICP)	Stable < -20°C	Liver function	Significant
Amino-terminal propeptide of type I procollagen (PINP)	Stable < -20°C	Liver function	Significant
Hydroxyproline, total and dialyzable (OH-Pro; OHP)	Stable < -20°C	Liver function, diet, inflammation (e.g., C1q)	Significant
Pyridinoline (PYD; Pyr)	Stable < -20°C	Liver function, active arthritis	Significant
Deoxypyridinoline (DPD, d-Pyr)	Stable < -20°C	Liver function	Significant
Carboxy terminal crosslinked telopeptide of type I collagen (ICTP)	Stable < -20°C	Renal function, liver function	Significant
Carboxy terminal crosslinked telopeptide of type I collagen (CTX)	Stable < -20°C	Renal function, liver function	Significant
Amino-terminal crosslinked telopeptide of type I collagen (INTP, NTx)	Stable < -20°C	Liver function	Significant
Hydroxylysine glycosides (Hyl-Glyc)	Stable < -20°C	Liver function	Significant
Bone sialoprotein (BSP)	Stable < -20°C	Renal function, liver function	Significant
Tartrate-resistant acid phosphatase (TRAP)	Unstable > -80°C	Hemolysis, blood clotting	Negligible
Free γ -carboxyglutamic (GLA)	?	Diet (e.g., vitamin K), blood clotting	Negligible

C-terminal (CTX) collagen type I telopeptides, while C-terminal telopeptide in serum (ICTP) loses up to 12% of the signal when stored at room temperature for 5 d (117). The stability of glycosylated hydroxylysine residues has not been fully characterized yet, but it may be necessary to add boric acid to preserve the urine samples. The activity of serum tartrate-resistant acid phosphatase (TRAP) declines rapidly during storage at room tem-

perature or even at -20°C but is stable when stored at -70°C or lower (114). Multiple freezing-thaw cycles usually have a deleterious effect on the serum TRAP activity. In contrast, serum levels of bone sialoprotein (BSP) appear rather stable, both at room temperature, 4 and -20°C , and have been shown to not change significantly during repeated freeze-thaw-cycles (107). However, when samples are being exposed to temperatures above 30°C , an increase in signal is usually seen with the RIA.

Some assays and marker components are sensitive to hemolysis of the sample, resulting in results that are either too low or too high. This is usually the case for osteocalcin and bone sialoprotein, but has also been described for TRAP and some other serum markers.

In general, random samples can be used for measurement of most urinary parameters (except urinary calcium, which always requires a 24-h sample). For convenience, measurement of bone turnover markers is usually performed either in first or second morning voids, or in 2-h collections. In each case, values need to be corrected for urinary creatinine, which introduces additional preanalytical and analytical variability. As an alternative, a 24-h urine collection can be ordered and the daily excretion rate of the marker determined.

The only bone turnover marker that is markedly affected by dietary influences is urinary hydroxyproline. Urinary levels of hydroxyproline will rise considerably after the ingestion of hydroxyproline-rich foods, such as meat or gelatin. Therefore, it is necessary to instruct patients to keep a collagen-free diet for at least 24 h before collecting their urine for hydroxyproline measurements. However, free and total DPD, NTX and CTX are all affected by ingestion of large amounts (100 g) of pure gelatin (119).

The effect of acute exercise immediately or shortly before phlebotomy for bone turnover markers has been studied with some markers appearing to rise by as much as 30–40% of their baseline value, others seem to be unaffected by these activities (140).

Biological Aspects of Preanalytical Variability

Diurnal variation appears not to be affected by age, menopause, physical activity or season. Although in postmenopausal women bone turnover is higher than in premenopausal women, the circadian variation is similar for both pre- and postmenopausal women and, thus, is not influenced by sex hormones (121–123). The etiology of diurnal variations is unknown. Several hormones, such as parathyroid hormone, growth hormone, or cortisol show diurnal changes and may therefore be involved in the generation of diurnal changes in bone metabolism (124). Independent of this, there is wide agreement that controlling the time of sampling is crucial in order to obtain clinically relevant information from bone markers. Most biochemical markers show significant diurnal variations, with highest values in the early morning hours and lowest values during the afternoon and at night. This has been well documented for most urinary markers (118) and amplitudes usually vary between 20 and 30%. Serum markers usually show less pronounced changes during the day than urine-based indices. However, discrepant results have been reported for serum CTX. Wichers et al. (120) reported a daily amplitude of serum CTX of up to 66%, while others describe smaller changes.

Intra-individually, biochemical markers of bone turnover not only vary within a single day but in most cases also between consecutive days. This phenomenon is called between-day or day-to-day variability and is apparently due to genuine variations in marker levels and not to analytical imprecision. In general, serum markers show less day-to-day variability than markers of bone turnover measured in urine (125). The day-to-day variation in the urinary excretion of PYD and DPD, measured by HPLC and corrected for crea-

tinine, ranges between 16–26% (126). Similar results have been reported for free pyridinoline by EIA (7–25%), for the N-terminal collagen type I telopeptide (NTX, 13–35%), for the C-terminal collagen type I telopeptide (CTX, 12–35%) and for tartrate-resistant acid phosphatase (TRAP, 10–12%) (125,127). Day-to-day variability adds considerably to the total variation of biochemical markers of bone turnover and unlike diurnal variations, day-to-day variability cannot be controlled.

Bone turnover varies with the menstrual cycle with an overall amplitude of approx 10–20% (128,129). There is evidence to support the suggestion that bone formation is higher during the luteal than the follicular phase (128), whereas bone resorption is higher during the mid-follicular, late-follicular and early luteal phase (130). Cyclical changes in bone turnover have also been reported in postmenopausal women treated with sequential estrogen/gestagen regimens, showing decreases during estrogen treatment and increases during gestagen treatment (131). In premenopausal women with metabolic bone disease, menstrual variability should be taken into account, and the timing for sampling is probably best during the first 3–7 d of the menstrual cycle.

Bone turnover and its regulation seem to vary with seasonal changes. Some studies have shown that serum 25-OH vitamin D and urinary calcium are higher in summer than in winter, and that parathyroid hormone levels show inverse changes (132,133). More recently, seasonal changes were also described for markers of bone metabolism, with a 20–30% lower turnover rate in summer than in winter (134; Fig. 5). The increase in bone turnover during the winter period may be due, at least in part, to subclinical vitamin D deficiency.

During early childhood and then again during the pubertal growth spurt, biochemical markers of bone turnover are significantly higher than during adulthood (135). In girls, peak bone marker levels are observed approximately two years earlier than in boys, and estradiol seems to be the major determinant of the increase in bone turnover. In men between 20 and 30 yr of age, bone turnover markers are usually higher than in women of the same age bracket. After the age of 50, most bone turnover markers tend to increase with further ageing, but less in men than in women. In the latter, the age-related increase in bone turnover is more pronounced due to the menopause, when both bone resorption and formation increase by approx 50–100% (136,137).

A number of nonskeletal diseases have been shown to strongly affect bone turnover markers. These conditions mostly relate to impairments in the clearance and/or metabolism of the components measured. Thus, even moderate impairment of renal function (GFR 50 mL/min) has been shown to have significant effects on the serum levels of osteocalcin (138), of bone sialoprotein (109), and of the collagen type I telopeptides (NTX, CTX) (139).

Numerous factors influence bone turnover, but there are even more sources of variability that need to be taken into account when measuring bone turnover with biochemical markers. To minimize some of the limitations linked to preanalytical and analytical variability, standardized sampling and sample handling are mandatory to obtain reliable results. Controllable factors such as the mode of sample collection, sample handling and storage, diurnal and menstrual rhythms, pre-sampling exercise and pre-sampling diet should be taken care of wherever possible. Laboratories are encouraged to establish their own reference ranges and to use gender and age specific reference intervals. In order to further reduce variability, standardization of bone marker assays and routine proficiency testing programs are strongly recommended.

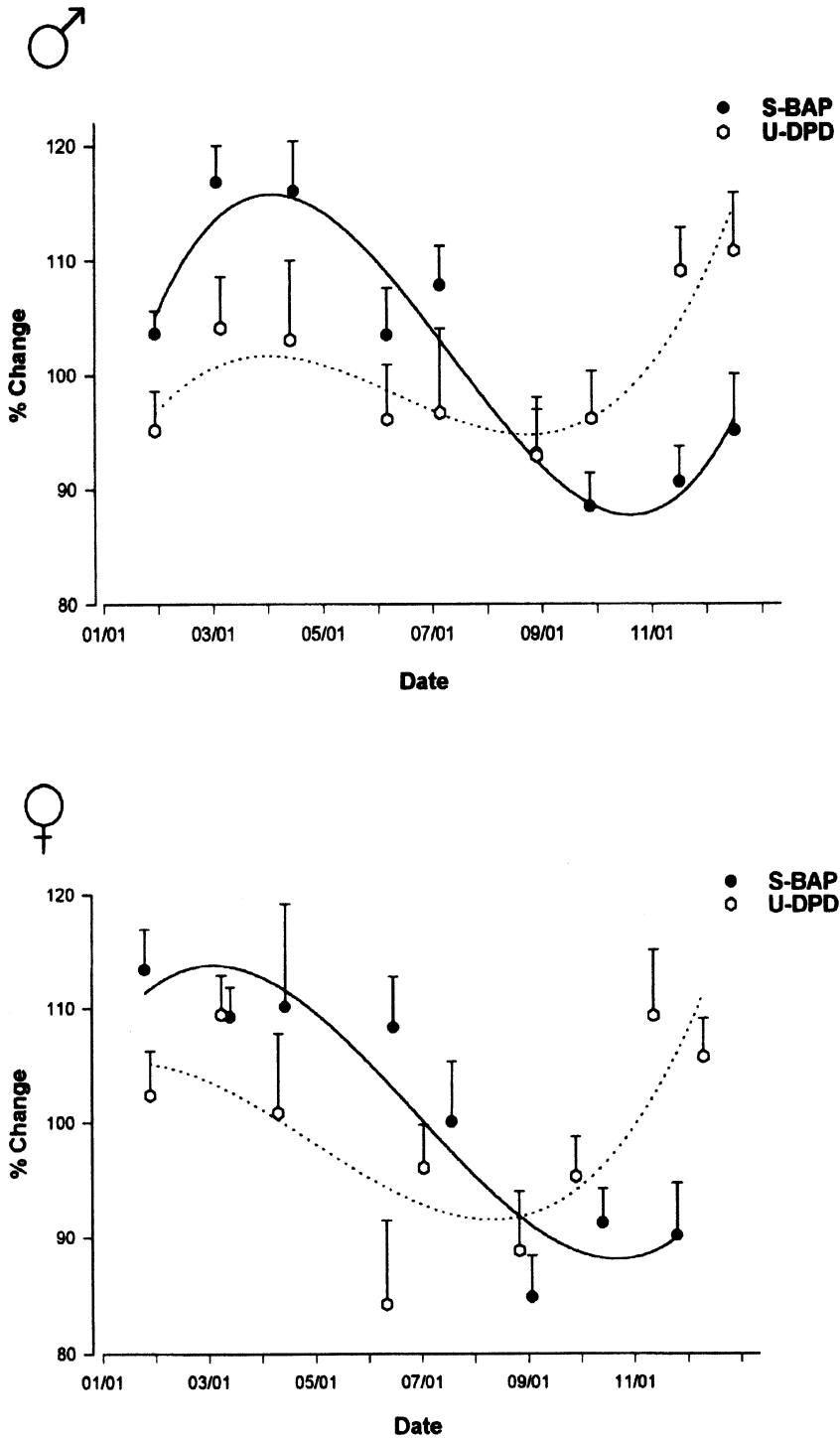


Fig. 5. Seasonal variability in S-BAP and U-DPD (univariate analysis). Values are given as the percent change from the annual mean (\pm SEM). Only those participants were included in this analysis who were present between January 1, 1992 and December 31, 1992, represented by the time course on the x-axis. The number and width of representative intervals of each bone marker were computed according to the method of Sturges, using $\Delta = (D^{\max} - D^{\min}) / (1 + 3.322 \times \lg n)$, in which Δ is the interval width, D^{\max} is the end of the analysis period (December 31, 1992), D^{\min}

Changes in marker measurements should always be interpreted against the background of the respective marker's total variability. As a rule, markers showing large changes in response to disease processes or interventions also show substantial degrees of variability.

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Fig. 5. (continued) is the beginning of the analysis period (January 1, 1992), n is the number of data points, and $1 + 3.322 \times \lg n$ gives the number of intervals. After computing mean values and weighing the number of data points in each interval, graphs were constructed using the least square curve fitting by means of a polynomial regression. *Data points* (mean values \pm SEM) are placed on the median of the respective interval (i.e., for S-BAP in males, January 25 represents the median of the first interval ranging from January 1 to February 15). Differences in the number and width of representative intervals between parameters result from missing laboratory values or elimination of extreme values in some instances (134).

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9

Bone Turnover Markers

Their Place in the Investigation of Osteoporosis

Richard Eastell, MD and Penny R. Bainbridge, BA

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INTRODUCTION

Bone turnover markers have been used in medicine for over 70 yr and have proven indispensable in metabolic bone disorders such as Paget's disease of bone. The perturbations in bone turnover in osteoporosis are small and early markers that are not specific to bone were not very useful in evaluating the disease. However, marker measures have recently been introduced that are more specific for bone. In this chapter, we will consider the approaches for the use of these markers in the evaluation of osteoporosis.

BONE TURNOVER MARKERS

Siebel has described the bone turnover markers in detail. They reflect osteoclastic bone resorption, and the activity of the osteoblast (Table 1). Most of the bone resorption markers are degradation products of type I collagen. They can be measured in the urine or serum. The bone formation markers reflect the synthesis of matrix proteins or enzymes by the osteoblast. They are usually measured in serum. These markers do not always behave uniformly in response to treatment. For bone formation markers, this may be explained by the shifts in protein production as the osteoblast matures, and by the different clearance mechanisms of the various markers. For the resorption measures, dis-

Table 1
Bone Turnover Markers

<i>Bone formation</i>	<i>Bone resorption</i>
Osteocalcin (OC)	Deoxypyridinoline (DPD)
Bone alkaline phosphatase (Bone ALP)	N-telopeptides of type I collagen (NTX)
Procollagen type I C-terminal propeptide (PICP)	C-telopeptides of type I collagen (CTX)
Procollagen type I N-terminal propeptide (PINP)	

Table 2
Sources of Variability in Bone Turnover Markers^a

<i>Controllable</i>	<i>Uncontrollable</i>
Circadian rhythm	Age
Menstrual rhythm	Gender
Avoidance of hemolysis and storage at appropriate temperature	Race
Diet	Diseases
Exercise	Drugs
	Fracture

^aSee ref. 1.

tinct patterns of responses may relate to differences in the bone-specificity of the markers and their metabolism by the kidney.

There have been recent improvements in the measurement of these markers. The introduction of assays for collagen N-telopeptide (NTX), collagen C-telopeptide (CTX) and Osteocalcin (OC) using automatic auto-analyzer devices has resulted in their widespread availability. Further, the intrinsic assay variability has decreased to less than 5%. Another technical advance is the introduction of point of care devices for measurement of urinary NTX and CTX. These devices allow for near patient testing and provide immediate feedback, thus helping in patient management.

SOURCES OF VARIABILITY IN BONE TURNOVER MARKERS

There are a number of sources of variability in the bone turnover markers. It is important to appreciate these in order to use markers effectively in clinical practice (1) (Table 2). The variability attributable to laboratory assay methodology is now quite low, especially with the introduction of automated immunoassay analyzers. The pre-laboratory variability can be divided into variability that is controllable (by the clinician) and those that are uncontrollable.

The controllable sources can be reduced by careful handling of the samples, and their collection at a fixed time of day, (e.g., second morning void urine collections [7–9 AM] or blood taken between 8 and 10 AM with the patient in the fasting state). The patient should be instructed not to drink alcohol excessively or exercise to exhaustion on the previous day. Day-to-day variation in marker level can be further reduced by making more than one measurement and using averaged results.

The use of separate reference ranges for men and women can improve assay result interpretation. Older women tend to have higher bone turnover markers than older men (2).

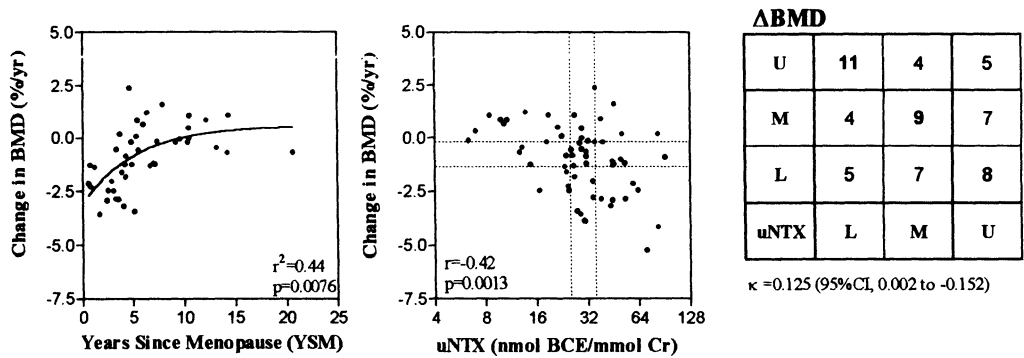


Fig. 1. The relationship between change in bone mineral density of the spine and bone turnover. (Left panel) Note how the bone loss is most rapid in the first 5 yr after the menopause. (Middle panel) The higher the rate of bone resorption (measured by NTX in a second morning void urine sample and expressed as a ratio to creatinine) the greater the rate of bone loss. The broken lines represent the boundaries between tertiles. (Right panel) The ability of bone resorption markers to classify individuals into upper (U), middle (M), or lower (L) tertiles of bone loss is poor with a kappa score of less than 0.2 making this approach unsuitable for use in the individual. Adapted from ref. 6.

REFERENCE RANGES FOR BONE TURNOVER MARKERS

The reference range is best established for women by including women whose skeletons have matured (older than 30 yr) and who have not yet reached the menopause. The reference ranges should be based on more than 100 subjects. Separate reference ranges are needed for children and for men. Most bone markers have a skewed distribution and so the reference ranges needs to be obtained after log-transformation of the data or using nonparametric statistics.

PREDICTION OF RATES OF BONE LOSS AND FRACTURE RISK

High levels of bone turnover markers are associated with high rates of bone loss in postmenopausal women. This relationship is stronger in women in the first five years after the menopause than in older women. However, the relationship is not strong enough to be useful in the individual woman (3–6) (Fig. 1).

There have been several prospective studies of the relationship between bone turnover markers and fracture risk in postmenopausal women (7–9). These have included risk of hip, spine and nonspine fracture. Bone resorption markers above the premenopausal reference range were associated with a doubling in the risk of hip fracture. This association is as strong as that for a one standard deviation decrease in proximal femoral bone mineral density (BMD). Thus, a low BMD or ultrasound measurement (of the heel) and a high bone resorption marker puts a woman at particularly high risk of fracture (13) (Fig. 2). The increase in risk of fracture with high bone resorption markers appears independent of bone mineral density. High bone turnover may increase bone fragility by an increase in the number of remodeling sites on the surface of bone that act as stress risers (12). The relationship of fracture risk with bone formation markers has not been so clear (7,8,10,11).

DIAGNOSIS OF PRIMARY AND SECONDARY OSTEOPOROSIS

Bone turnover markers may be normal or increased in primary osteoporosis and so they cannot be used as a diagnostic test. There is a weak negative correlation with bone

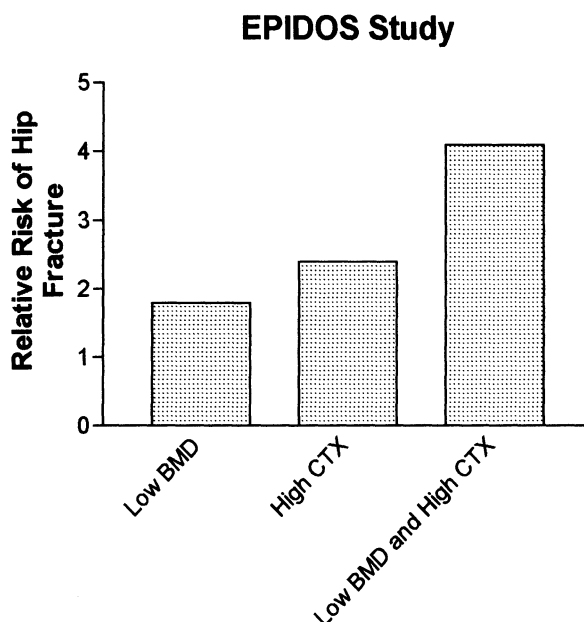


Fig. 2. The risk of hip fracture is higher in women with low hip bone mineral density ($T < -2.9$), high urinary CTX ($T > 2$), and even higher with both low bone mineral density ($T < -2.5$) and high urinary CTX ($T > 2$).

mineral density, but this does not allow the prediction of BMD in the individual (14). Only about 25% of patients with primary osteoporosis have bone turnover marker levels above the reference range for young individuals (15).

Bone markers allow the identification of patients with lower rates of bone turnover. The bone density response to antiresorptive therapy tends to be slightly less in these individuals (16), but it is not clear that they have a lesser improvement in their risk of fracture in response to treatment. Thus, low bone turnover markers cannot be used yet in the selection of appropriate therapy.

High levels of bone turnover markers may indicate secondary osteoporosis and so encourage the search for underlying diseases. For example, bone turnover markers are increased in about 50% of patients with primary hyperparathyroidism and are also increased in thyrotoxicosis and in multiple myeloma, malignant bone disease (17,18). Whether marker measurements are cost-effective for the detection of secondary causes of osteoporosis is unknown.

MONITORING OF TREATMENT RESPONSE

Why Monitor Therapy?

Monitoring osteoporosis therapy has been suggested with two goals in mind: to identify nonresponders and, to improve adherence to therapy.

Therapeutic of nonresponse is difficult to define. Some authors have suggested that a decrease in BMD during therapy should be considered a nonresponse. Using this criterion, about 15% of patients taking HRT are nonresponders (19). However, it is possible that had these women not been treated they may have lost even more bone. The variance of rates of change in BMD is similar for women treated with HRT or

bisphosphonates as for controls (16). This suggests that in the setting of clinical trials true nonresponders are rare.

In clinical practice, there are situations that may result in a lack of response to osteoporosis therapies. For instance, patients who have no change (or a decrease) in BMD may have secondary osteoporosis. When the underlying disease is treated there should be an increase in BMD in response to treatment. Examples include osteomalacia (20) and thyrotoxicosis. Nonresponders might be identified using the least significant change approach (described in more detail below for bone turnover markers), however, the success of this approach has not been specifically evaluated.

Compliance with treatments for osteoporosis can be poor since there is rarely a reduction in symptoms and there may be adverse effects from medications such as HRT and bisphosphonates. The discontinuation of therapy with HRT and bisphosphonates at 1 yr can be as high as 50%. Many patients discontinue therapy within the first 3 mo. Monitoring treatment improves compliance, but this contention has yet to be tested.

What Advantages do Bone Turnover Markers Have for Treatment Monitoring?

Bone mineral density has been used for monitoring of treatment response (22). The disadvantages of this approach are: 1) the BMD response is slow, and it commonly takes one to two years for BMD to exceed the least significant change; 2) the spine is the best site for treatment monitoring as it has the greatest response with the best precision, but the spine is also commonly affected by degenerative changes in patients over the age of 60 yr; 3) the rate of loss in an individual with no treatment is unknown; in perimenopausal women or in patients starting corticosteroids, the loss is rapid, but in women with established osteoporosis there may be no bone loss. Furthermore, the test requires purchase of equipment and the employment of a suitable technician and so may only be available in specialized centers.

Bone turnover markers do not suffer from these limitations. The response to antiresorptive therapy is both large and rapid, with maximum suppression of bone resorption markers of about 50% within 3 mo of starting therapy. There is no systematic change in bone turnover markers if no treatment is given. There is no initial outlay for equipment; samples can be sent to a referral laboratory for marker assays or point of care measures can be used.

The bone markers also allow identification of suppression of bone turnover to below the reference range. The clinical significance of such "over suppression" is not yet known, but in experimental animals it may result in deterioration in the biomechanical properties of bone and the accumulation of microdamage (23).

Disadvantages of Bone Turnover Markers

The principal disadvantages to using bone turnover markers to monitor therapy are their variability and the inconvenience of sample collection. Some sources of variability can be minimised as described in the section on the practical use of the markers. Nonetheless, not all sources of variation can be controlled and thus variability must be taken into account in the interpretation of the results of marker measurements. For example, a recent fracture results in an increase in bone turnover markers and so care needs to be taken in documenting such an event (24,25). Physicians who use bone turnover markers need to be well briefed about all the sources of variability and the appropriate identification of change.

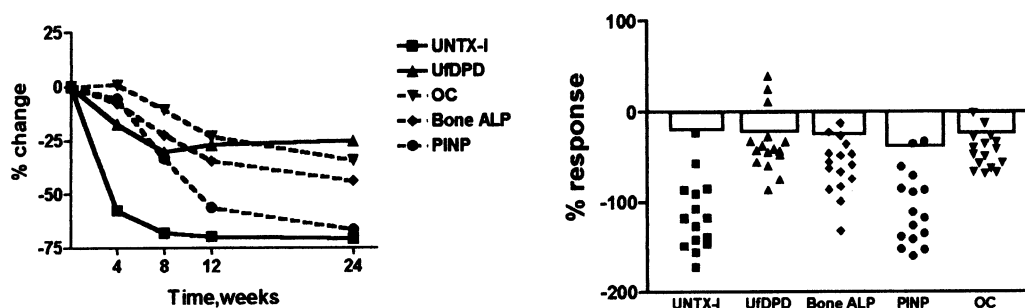


Fig. 3. The effect of alendronate therapy on bone turnover markers over 6 mo. Left, note bone resorption markers are suppressed within 4 to 8 wk of starting therapy; the decrease in bone formation markers is somewhat slower. Right, the boxes show the least significant change bounds for each marker. Most patients show a response to treatment at 6 mo. Patients entering studies such as this are highly selected; in clinical practice, patients may have secondary osteoporosis that prevents them responding to therapy, or they may not comply with instructions. Adapted from ref. 33.

Can Markers be Used to Monitor all Treatments?

The effect of antiresorptive therapy on bone turnover markers has been repeatedly described. The decrease in bone resorption markers is usually maximal by three months. A similar decrease in bone formation markers is fully established by 6–9 mo. The time course of the response to HRT may be slower than that of bisphosphonates, and the response to intravenous bisphosphonates (32) may be more rapid than to oral bisphosphonates (33) (Fig. 3). The size of the effect on markers differs between treatments: calcium and calcitonin (34) < raloxifene (35) < risedronate (36) and HRT (30,37) < alendronate (28,33). The fall in markers in response to calcium and calcitonin may be too small to be useful for monitoring treatment in practice.

There is less information on formation stimulating therapies. In response to parathyroid hormone (PTH) therapy, osteocalcin (a bone formation marker) increases by 60% at 1 mo, whereas NTX (a bone resorption marker) increases by the same amount by 6 mo (38).

Can Markers be Used in Both Primary and Secondary OP?

Antiresorptive therapy in patients treated with glucocorticoids results in changes in bone turnover markers with a time course similar to that observed in patients with primary osteoporosis (39,40). It is not yet known whether the variability in marker response is also similar, but comparable findings were noted in preliminary studies (41).

Validation of Bone Marker Response as a Method for Assessing Treatment

Several studies have examined the relationship between change in bone turnover markers and change in BMD during trials of osteoporosis therapies. The results have been inconsistent. In some trials a weak relationship between change in bone turnover and change in BMD has been noted (29,30) although this finding is not universal (5). Other studies inappropriately combined treatment and placebo groups in the same analysis (27,28). The relationship between rates of change in BMD and markers is expected to be weak because there is variability not just in the estimate of change in bone turnover but also in that for bone mineral density. It has been estimated that the maximum correlation coefficient possible given the variability of the measurements is about 0.3.

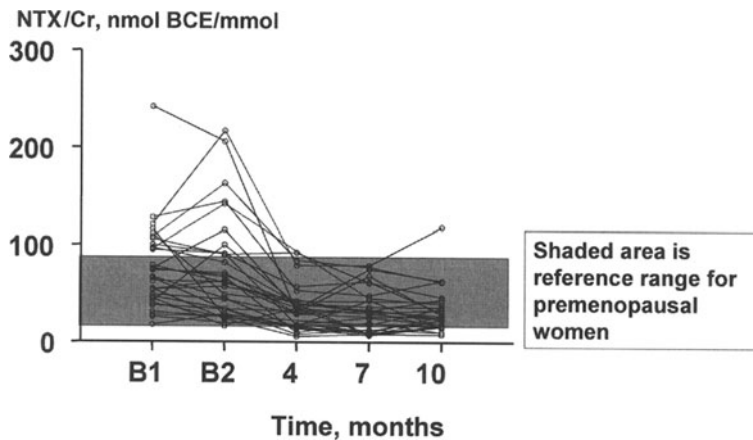


Fig. 4. NTX was measured in the second morning void urine sample in 49 patient with osteoporosis (46) on two occasions at baseline (B1 and B2) and then after 4, 7, and 10 mo on treatment with HRT or bisphosphonates. NTX levels vary before starting treatment, and there is an early decrease in NTX after starting treatment. The maximum effect is seen by 4 mo in most patients. NTX levels are in the lower half of the premenopausal reference range in most subjects after 10 mo of treatment.

It is similarly uncertain whether the change in bone markers reflects a reduction in the risk of fracture in the individual. An increase in total hip BMD in response to alendronate is associated with a decrease in the risk of vertebral fractures (26), but similar analyses have not been performed of the relationships between changes in bone markers and fracture risk.

HOW TO INTERPRET RESULTS/ASSESSING CHANGE IN MARKER LEVELS

A number of approaches have been used to confidently identify change in marker levels. The least significant change approach will be described in detail (33,37).

The least significant change method is widely used in medicine. It requires knowledge of the variability of a measurement over a relevant period of time in the absence of treatment, usually expressed as a coefficient of variation (CV). If one measurement is made at baseline, and change is to be assessed in either direction (increases as well as decreases) at a significance level (p of 0.05), then the following formula is used:

$$\text{Least significant change (LSC)} = 1.96 \times \text{square root of } 2 \times \text{CV} = 2.77 \times \text{CV}$$

If duplicate measurements are made at baseline and again at follow-up, and the only change of interest is in one direction (e.g., a decrease) (i.e., one-tailed) for a p -value of 0.05, then:

$$\text{LSC} = 1.65 \times \text{CV}$$

For example, in our clinical practice, we make two marker measurements at baseline and again at 4 and 7 mo on treatment (Fig. 4). The means of the two baseline and of the two follow-up measurements are calculated and if the percentage change (calculated as the difference between baseline and follow-up $\times 100$ /average of baseline and follow-up) exceeds the LSC a difference is considered to be confidently established. The CV in a clinical practice setting is 33% for NTX, and so the least significant change that we can detect is 54%.

A limitation to this approach is that it is simply based on a statistical consideration and not based on some external standard, e.g., the change in BMD. Also, $p = 0.05$ may be too stringent for decision making in clinical practice.

Other approaches have been proposed:

1. The T-score approach. This method is analogous to that used for BMD measures. The young normal reference range for a marker measurement is established (e.g., in premenopausal women between ages 30 and 45 yr) and the object of therapy is to ensure markers levels similar to those in young normal women (i.e., a marker T-score of 0) (28). A limitation of this approach is that many women with osteoporosis have a marker T-score close to 0 before therapy.
2. A combination of the change in marker and the final bone marker result. For example, a goal might be a decrease in serum bone alkaline phosphatase (measured by IRMA) of more than 20% and a final value below $8 \mu\text{g/L}$ (42). Again, patients may have low values to begin with, and a change of 20% may not exceed the least significant change threshold. Further, this approach is difficult to display graphically.
3. A specificity of 90% for the prediction of change in BMD. Here the BMD change is categorised into response and no response and published information concerning both control and active treatment groups are considered. The change in bone markers predictive of a BMD response with a specificity of 90% is considered the cut-off for a confident change in bone turnover. For example, 90% of BMD non-responders to alendronate have been described to have a decrease in bone alkaline phosphatase of less than 38% . Limitations of this approach include that the analysis depends on responses reported in both controls and treated patients, and that it assumes that BMD responses explain the antifracture effect of therapy. Both of these assumptions may be incorrect, as discussed above.

PRACTICAL IMPLEMENTATION

Who to Monitor?

In fact, there is no consensus as to whether any patient should be monitored with marker measurements during osteoporosis therapy. In the United States, National Osteoporosis Foundation guidelines do not support this use (44), while the International Osteoporosis Foundation guidelines do suggest this use for markers (45)! At present, we use markers for monitoring patients with established osteoporosis. If the case can be made that response assessed with marker measurements is associated with reduction in fracture risk and that compliance is enhanced by monitoring change in marker levels, it may also be reasonable to monitor all patients starting treatment for osteoporosis.

Sample Collection

Before osteoporosis therapy is begun, we obtain duplicate measurements of both formation and resorption markers and repeat those measurements at 4 and 7 mo. Blood tests

Fig. 5. (*opposite page*) Changes of bone turnover markers in response to HRT over 1 yr. This postmenopausal woman had osteoporosis secondary to rheumatoid arthritis and glucocorticoid therapy. She had two baseline collections of blood and urine (B1 and B2) and the mean was calculated (mB1B2). Note the elevation of two of the three markers. She was then treated with continuous combined hormone replacement therapy (estradiol valerate 2 mg/d, norethisterone 700 mcg/d) and samples were taken at 4, 7, and 10 mo (v1, 2, 3). The left panels show the actual values of bone turnover markers with premenopausal reference intervals shown by the broken lines.

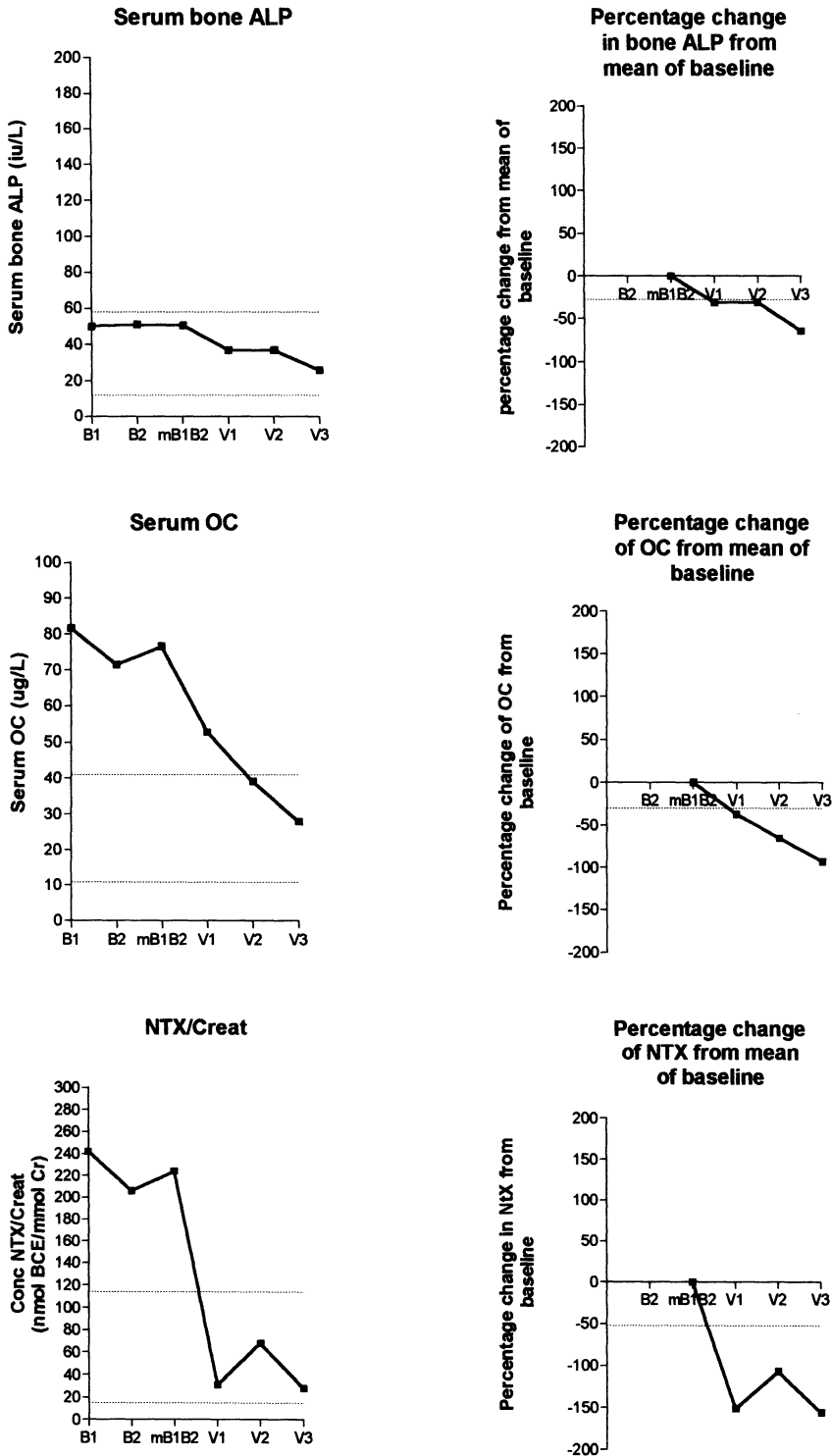


Fig. 5. (continued) The right panels show the percentage change and the broken lines indicate the least significant change (for double measurements, one-tailed, $p = 0.05$). The percentage change is calculated with the mean of the initial and later result in the denominator so that the maximum increase or decrease is 200%. A clear response of all three markers was seen and the lumbar spine BMD increased by 9% at 1 yr.

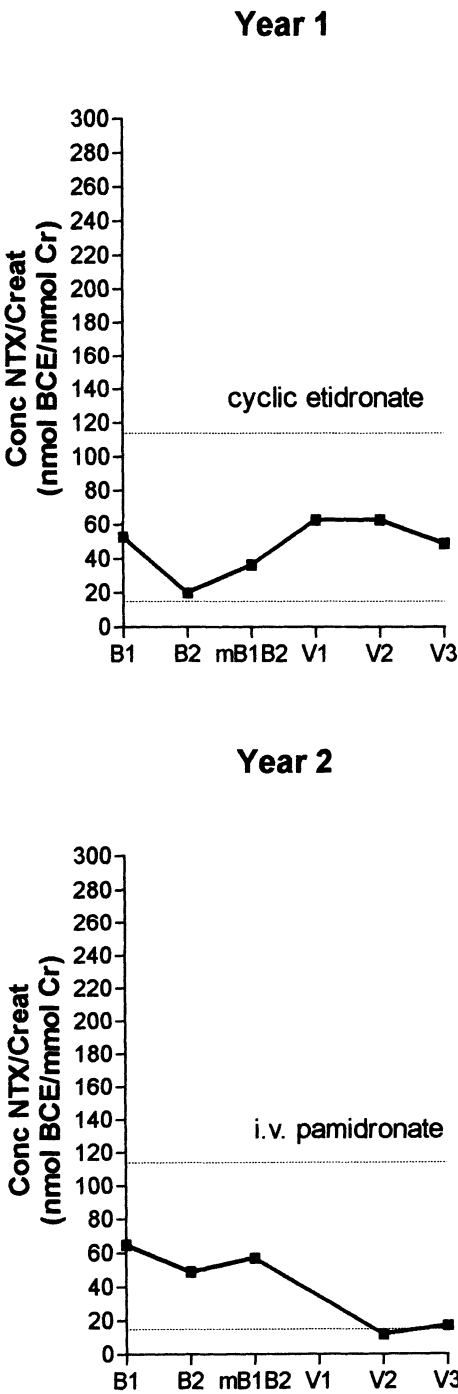


Fig. 6. An example of a nonresponder. This middle-aged man abused alcohol (18 U/d) and during the first year of treatment with cyclic etidronate showed no bone turnover response and a non-significant decrease in spine BMD of 1.6%. In the second year he was treated with pamidronate 60 mg given intravenously every 3 mo. He had a clear response in NTX levels with an increase in spine BMD of 6.2%.

are taken at any time of the day; urine collections are made between 9 and 11 AM. We take this relaxed approach to blood collections because bone alkaline phosphatase (ALP) and OC do not have large circadian rhythms. The urine collection may also be a second morning void, or a 24-h collection, but the timed morning collection fits best into our practice.

Which Markers?

BASELINE

We have found bone alkaline phosphatase to be particularly useful for the detection of Paget's disease of bone and secondary hyperparathyroidism (osteomalacia, malabsorption syndrome). We have found serum osteocalcin to be useful in glucocorticoid-induced osteoporosis and PINP and bone resorption markers to be useful for malignancy.

FOLLOW-UP

The telopeptide markers and PINP tend to show greater responses to antiresorptive therapy and lower LSC thresholds. We prefer the automated analysers in view of their reliability and high precision. In the future, there may be a role for point of care devices as these allow immediate reporting of results.

How to Display Results

The results of marker measurements are graphed to provide two kinds of information. The percent change in marker level in response to therapy is calculated as described above and is plotted as change from baseline (Fig. 5 [right column]). The graph includes the threshold that indicates least significant change. Also, we graph the absolute marker results with an indication of the premenopausal (or young) reference range (Fig. 5 [left column]). The graphical display of the data is easily understood by most patients.

Action to Take if There is No Response

In our experience, a decline in urinary NTX excretion occurs in about 65% of patients treated with HRT or bisphosphonates. In the remainder, we enquire about the adherence to treatment and reconsider whether secondary osteoporosis is present or whether there is some other case of high bone turnover such as a recent fracture. If there is no BMD response at 18 mo we then consider changing the dose of treatment or changing to an alternative treatment or route of administration (Fig. 6).

ADDENDUM

Since the preparation of this article, Bjarnason et al. (Osteoporosis Int., 2001) have reported that those women treated with raloxifene who had the greatest decrease in osteocalcin had the lowest risk of vertebral fracture. There was no association of fracture risk and change in bone mineral density. This supports the idea that bone turnover markers may help explain the reduction in fracture risk with antiresorptive agents such as raloxifene.

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10

Mechanical Influences on Bone Mass and Morphology

Investigating How Exercise May Regulate Adaptation in the Skeleton

Stefan Judex, PhD and Clinton Rubin, PhD

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EXERCISE INFLUENCES ON BONE MORPHOLOGY IN CLINICAL SETTINGS

Introduction

A link between mechanical forces and skeletal morphology was recognized by Galileo Galilei in the early 1600s (1). Two centuries later, the interdependence of form and function was proposed to explain the intricate architecture of trabecular bone morphology and the changes in bone mass with altered mechanical demands (2–4). An increase in the level of physical activity caused an increase in bone mass, and reduced physical activity stimulated bone loss. Since these early observations, this relation between changes in function and altered bone morphology has been studied extensively with the goal of defining the mechanical parameters which give rise to the adaptive response.

Simultaneously, many studies have attempted to quantify the effects of exercise regimens on bone mass and morphology. While some studies have provided encouraging data, the large majority of data have been equivocal. Although there is little argument that bone can readily respond to mechanical stimuli, it is more than likely that the inconclusive results reflect our limited understanding of which specific component of the mechanical signal is perceived as osteogenic by the resident bone cell populations (osteocytes, osteoblasts, and osteoclasts), thus crippling our efforts to design the “opti-

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mal exercise intervention.” Many critical questions have yet to be addressed, such as: should the exercise protocol incorporate large loads or could they be small if they are applied rapidly? For how long does one have to exercise to obtain maximal benefits? Can exercises be designed to focally deposit new bone at skeletal sites most prone to fracture? By identifying critical aspects of bone’s loading environment, it is hoped that specific exercise interventions or devices which induce regulatory mechanical signals can be developed to prevent the bone loss which leads to osteoporosis.

Here, we briefly overview exercise effects on bone (*see* Chapter 11 for more detail), define the functional mechanical environment of bone and demonstrate that the ability of physical signals to influence bone morphology is strongly dependent on the character of the signal. We will then show that an improved understanding of which components of bone’s mechanical milieu are perceived as osteogenic can be harnessed for the design of a biomechanical prophylaxis against bone loss. Further, we will provide preliminary evidence that extremely low-level but high-frequency mechanical signals, an example for the design of such an prophylaxis, are osteogenic and capable of inhibiting postmenopausal bone loss.

Can Exercise Increase Bone Mass?

Cross-sectional studies consistently reveal that bone morphology can change markedly in response to long-term exercise. For example, in professional tennis players, the cortical thickness of the humerus in the extremity which holds the racket can be up to 45% larger in the male and 30% larger in the female compared to the humerus in the nonplaying arm (5). Similar bone hypertrophy has been reported in feet of classical ballet dancers (6). Other cross-sectional studies have related a variety of different kind of physical activities to increased bone mass including soccer, weightlifting, speed skating, squash, dancing/gymnastics, and a generally higher level of physical activity (7–10). Self selection bias, however, cannot be discounted in cross-sectional comparative studies and the results from longitudinal prospective studies may provide more reliable information on how bone responds to exercise. Further, the specific aspect of the sport which is relevant to bone is difficult to define: is it because the athletes practice for so long? So hard? So fast?

Very few prospectively designed exercise trials demonstrate that exercise produces large increases in bone mass. Intense exercise in young army recruits (11) stimulated large increases in bone mineral density (BMD) but it was unclear whether this was an adaptation of the skeletal system, or the early pathological stages of stress fractures. In premenarchal girls, a high-impact strength building regimen increased bone BMD over a 10-mo period, in particular at the femoral neck where the increase in exercisers was 12.0% compared to 1.7% in controls. Unfortunately, the large majority of longitudinal studies has found only modest (12–16) if not negligible (17–20) increases in bone mass. For instance, in a 12-mo trial young adult females unilaterally performed high-resistance strength training (18). The training significantly increased muscle strength but bone mass was unaffected by the exercise intervention.

When results from cross-sectional and prospective exercise studies are stratified for the different exercise interventions used (e.g., gymnastics, running, or swimming), the results only hint that certain types of exercise are more effective than others in stimulating bone formation or inhibiting resorption. Some studies suggest that high-load, high-impact exercises using few repetitions are superior to those exercises using low loads and high repetitions (21,22). Swimming, as an example of a low-load high-repetition exercise has often failed to demonstrate significant skeletal benefits (22,23). Nevertheless,

some reports indicate that aerobic exercise (including swimming) is also capable of eliciting an adaptive response in bone (24–27), while a few strength exercise regimens failed to increase BMD (20,28). Comparative studies often find only modest differences among different modes of exercise, and the largest differences are frequently observed between those who exercise and controls, rather than between athletes subjected to differing exercise programs (12,24,27).

Constraints in Clinical Studies

Numerous clinical exercise studies have attempted to relate adaptive changes in bone to specific aspects of a particular exercise regimen including exercise mode (e.g., running, swimming, weightlifting), intensity (e.g., percent of maximal heart rate), duration, and frequency. Not surprisingly, none of these aspects has been successfully linked to exercise related changes in bone formation or resorption as this categorization fails to include the local mechanical milieu engendered by the specific exercise protocol. Further, bone adaptation is site-specific in nature at both the organ and the tissue level and, therefore, is only weakly related to parameters that reflect whole body phenomenon, such as exercise mode or intensity. At the organ level, bone adaptation is site-specific because only those bones that are subjected to changes in relevant mechanical stimuli respond with adaptive changes. This is particularly obvious in tennis and squash players who display bone hypertrophy in the playing extremity but not in the contralateral extremity. Similarly, in a longitudinal prospective study of postmenopausal women, unilateral strength exercise increased BMD in the mechanically stressed arm while the contralateral arm was unaffected (29).

Moving from the organ to the tissue level, it is critical to realize that not all bone cells within an adapting bone respond equally to the exercise related stimulus as some surfaces change their osteoblastic or osteoclastic activity while other surfaces remain unchanged. An example of these focal changes is a study in which adult roosters were exercised on a treadmill for 3 wk while a second group of roosters served as sedentary controls (30). Three weeks of high-speed running for 9 min/d activated previously quiescent periosteal middiaphyseal surfaces in the tarsometatarsus of exercised roosters, while no periosteal activity was observed in control roosters. Periosteal activation in exercisers occurred primarily at the medial and antero-lateral aspects of the middiaphysis while activation at anterior and posterior surfaces was minimal. The total percentage of activated surface in exercisers was only 23% of the entire periosteal surface.

The focal nature of bone's adaptation to mechanical stimuli prompts the question of whether the assays used to evaluate potential skeletal benefits in clinical studies are sensitive enough to detect site-specific changes and, further, whether alterations in bone mineral density accurately reflect the structural result of bone's adaptive response to exercise. The current gold standard for evaluating bone's response in clinical settings is dual energy x-ray absorptiometry (DXA). This technique is easy to use, noninvasive, and quantifies bone mineral content, BMC, in grams (g). A potential limitation of DXA, however, is that it provides only a two dimensional quantitation of bone mineral density BMD (g/cm^2), by normalizing BMC to the scanned area. As such, BMD not only dilutes focal changes in BMC within this area but also ignores the depth of the scanned region.

This incomplete correction for size causes DXA to underestimate true bone mineral density in people with small bones and to overestimate bone mineral density in people with large bones (31). Further, DXA cannot detect changes in bone quality. Bone quality is a

measure of the structural efficiency of the extracellular matrix (e.g., the architectural alignment of trabecular bone). Neglecting bone quality poses a critical limitation for the assessment of bone integrity, as bone is a complex structure in which mechanical function cannot be explained just by its mass. Fluoride treatment, for instance, evokes large increases in BMD at certain doses, yet may deteriorate the structural integrity of the bone due to poor mineralization of the bone tissue (32). Consequently, the addition of only a small amount of new bone that is well mineralized and deposited at a mechanically relevant site may have a more beneficial impact than the addition of a large amount of bone that is mechanically inferior.

The difficulty of quantifying the local mechanical environment during exercise in humans as well as the limited ability to quantitate beneficial effects of exercise on the human skeleton are two of the reasons that many investigators have turned to animal studies. Using animals, bone's mechanical milieu can be quantified and controlled. Further, several assays are available to determine changes in bone mass at the organ as well tissue level (e.g., μ CT, histomorphometry, mechanical tests, ultrasound). These tests can detect site specific and focal changes in bone formation/resorption and can also distinguish between altered bone quality and bone quantity in different directions within the bone. Taken together, these assays help to define the relation of specific components of bone's mechanical milieu to changes in bone quantity and quality, thus enabling the identification of distinct aspects of the functional loading environment that play a critical role in bone adaptation.

BONE'S MECHANICAL ENVIRONMENT

The inability of parameters such as exercise mode or intensity to predict changes in bone quantity or quality emphasizes the need to search for parameters involved in exercise related bone adaptation at the level of the bone itself. To this end, the mechanical environment in bone during functional activities has been characterized. This characterization is difficult, as during functional activities bones are not only loaded axially but are also subjected to moments produced by torsion and bending (Fig. 1B). A bending moment is produced by force couples that bend a structure—an effective way of snapping a stick that is placed over a thigh and pushed down on either end. Torsional loading twists the bone about its axis.

These forces and moments applied to a bone produce a complex mechanical state in the bone matrix which is quantified both spatially and temporally. Mechanical *strain* (ϵ) is the most common measure to quantify deformations in the bone matrix and is expressed as a change in length (ΔL) normalized to the original length (L) of any given specimen ($\epsilon = \Delta L/L$) (Fig. 1A). Thus, strain is a dimensionless measure and is commonly expressed in microstrain (10^2 strain); 1% deformation = 1.0% ϵ (or strain) = 0.01 ϵ = 10,000 $\mu\epsilon$ (or μ strain). Strains can be divided into normal and shear strains with normal strains producing volumetric changes and shear strains producing geometric angular changes (Fig. 1B). Spatial *strain gradients* give information about how strain magnitude changes across a volume of tissue. These gradients can be calculated for different directions within the bone matrix (e.g., circumferential, radial, longitudinal). *Strain rate* takes the temporal component of mechanical loading into account and is used to describe how quickly strain changes within a given time; the larger the strain rate the quicker the load (or moment) was applied to the bone. *Strain frequency* gives information about how many strain events occur within a certain time frame; 1 Hz = one cycle per second.

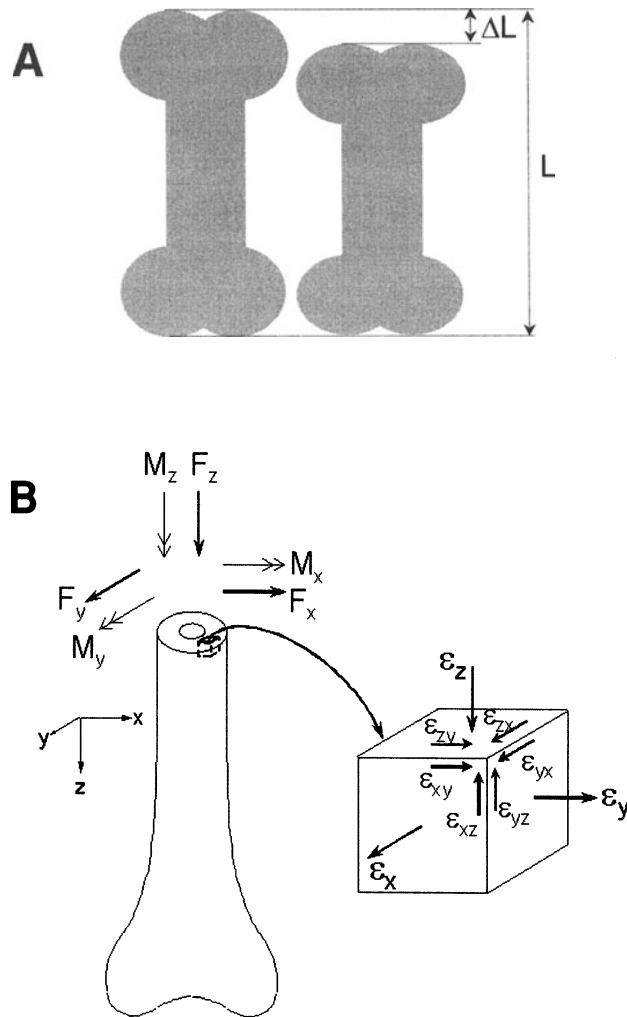


Fig. 1. (A) Illustration of the calculation of strain. Assuming that the original length of the bone is “L” and the bone is compressed by “ ΔL ”, then strain is determined as the ratio of deformation ΔL to the original length L. Thus, strain represents a normalized deformation and is dimensionless. (B) Functional load environment acting on a middiaphyseal section of a long bone consisting of bending moments (M_x , M_y), a torsional moment (M_z), shear forces (F_x , F_y), and an axial force (F_z). Many functional activities induce diaphysial strains that are generated primarily by moments. Diaphysial moments are influenced by joint and muscular forces and moments as well as by the degree of bone curvature. Although strain, as illustrated in (A) is a conceptually simple parameter when measured in only one direction, it is critical to realize that functional activities produce a complex state of strain in any given cube within the bone structure. This strain state can be characterized by normal strains (ϵ_{xx} , ϵ_{yy} , ϵ_{zz}) acting perpendicular to the faces of the cube which compress or elongate the cube. Shear strains (ϵ_{xy} , ϵ_{xz} , ϵ_{yz} , etc.) that are primarily produced by shear forces and torsional moments cause an angular change in cube geometry. Adapted from Judex S, Whiting WC, Zernicke KF Bone biomechanics and fracture, in Biomechanics in Ergonomics (Kumar S, ed.), Taylor & Francis, London, UK, 1999.

The magnitude of these parameters can be determined during functional activities when strain gages are surgically implanted onto a bone surface. While these gages only record

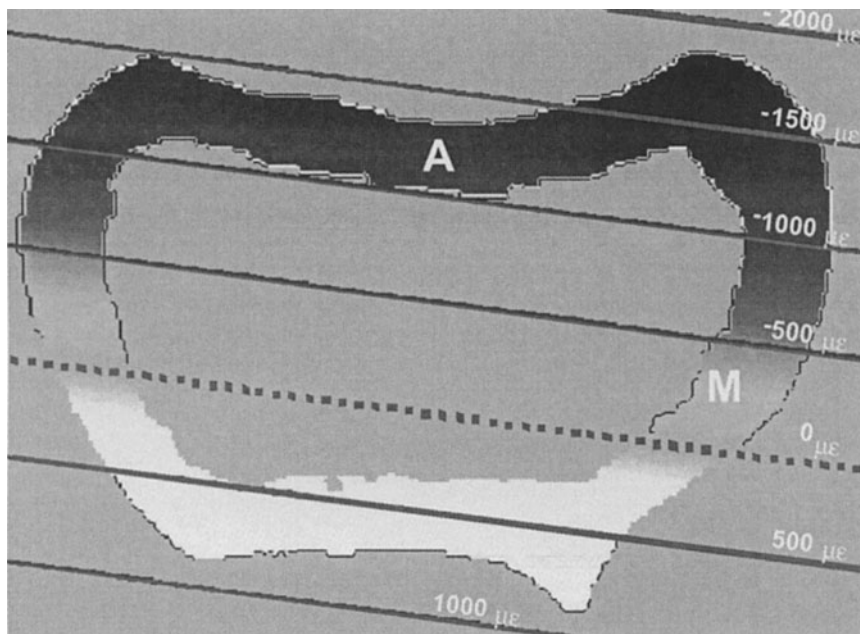


Fig. 2. Distribution of longitudinal normal strain superimposed upon a midthiaphyseal tarsometatarsal section in a rooster running at a treadmill speed of 1.7 m/s. Bending moments generated regions of large compressive strains (~ 1500 – $1800 \mu\epsilon$) at the anterior cortex (shaded dark) and regions of large tensile strains (~ 500 – $800 \mu\epsilon$) at the posterior cortex (white). The neutral axis (zero strain) is dotted with strain isopleths running parallel to it. This distribution was determined during mid-stance phase when peak strains were induced but the manner of loading (i.e., position of the neutral axis) did not change throughout stance phase or with changes in exercise mode (*see* also Fig. 3). A, anterior; M, medial.

deformation from the specific sites to which they are attached, mechanical models such as beam theory can be used to extrapolate measured deformations to other sites within the bone. This approach works well for cortical bone because of its accessibility to strain gages, but is exceedingly difficult for trabecular bone where sophisticated finite element models with numerous loading assumptions are needed to estimate the strain state. In cortical bone, strain gages have been used to quantify activity related bone strains in a great variety of species including humans (33,34), dogs (35,36), primates (37,38), roosters (39,40), horses (41,42), sheep (43), and rats (44). From these strain gage data, there are a number of critical observations to be made regarding the structural demands made on the skeleton.

Regardless of species or skeletal site, vigorous physical activity induces similar peak strains in the 2000–3000 $\mu\epsilon$ (microstrain) range (45–47). Since fractures usually occur at strains 2–3 times greater, this indicates that bone loading and architecture is finely tuned to achieve a safety factor with respect to the level of bone strain at which permanent deformation (damage) is induced. Whether measured in a galloping horse (metacarpal), running human (tibia), flying goose (humerus), trotting sheep (femur), or chewing macaque (mandible), this “dynamic strain similarity” suggests that skeletal morphology is adjusted in such a way that functional activity elicits a very specific (and perhaps beneficial) level of strain to the bone tissue (48). This similarity in peak strain magni-

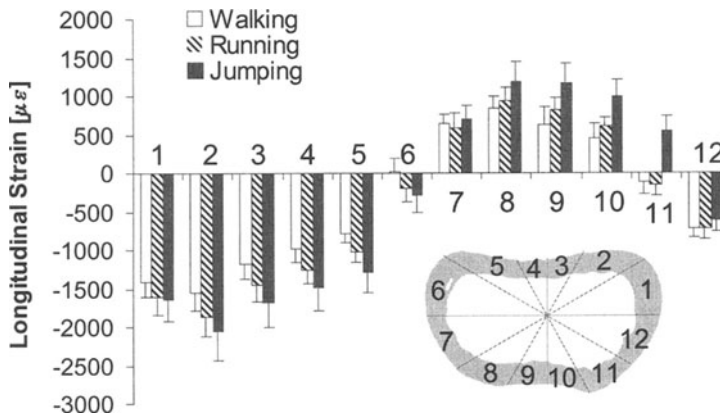


Fig. 3. Peak longitudinal strain determined in each of 12 sectors subdividing a middiaphyseal section when growing roosters were subjected to either slow walking, high-speed running, or drop jumps. Peak strains increased from walking to running and jumping, yet the relative distribution across the middiaphysis was similar for the three activities, indicating an extremely stable strain environment independent of exercise mode. Numbers along the abscissa refer to the sector numbers in the inset (mean \pm SE).

tudes also supports the hypothesis that achieving a specific level of peak strain is an adaptive goal that bone as an organ strives for.

During locomotion, bending is the dominant form of loading in the middiaphysis of limb bones; more than 85% of the strain measured in diaphyseal long bones is accounted for by bending moments (46). These middiaphyseal bending moments are caused by bone curvature as well as applied bending moments and generate compressive strains on one side of the cortex and tensile strains on the opposite side (Fig. 2). The dominance of bending in the functional loading environment is somewhat unexpected as far less bone mass would be required to support the same loads if the bone were loaded axially. Nevertheless, bending may be beneficial to bone as it creates a non-uniform strain environment that is more diverse than uniform compression, thereby enabling signaling pathways that could not be generated by uniform loading. Perhaps more importantly, bending causes consistent, predictable loading conditions in which specific regions within the cortex are loaded in a similar fashion independent of the functional activity. To demonstrate this stability of the functional strain environment, roosters were subjected to three distinct loading environments engendered by treadmill walking, treadmill running, or drop-jumps in which roosters were released from a 50–60 cm height. Tarsometatarsal middiaphyseal strain magnitudes and distribution were determined via strain gages and linear beam theory (49). We found that peak compressive strains produced by these three activities increased from $-1570 \mu\epsilon$ for walking to $-1870 \mu\epsilon$ for running and $-2070 \mu\epsilon$ for jumping. Despite this increase in peak strains, the relative distribution of strain across the middiaphysis remained remarkably stable across the three activities (Fig. 3).

With bending causing tension on one surface and compression on another, the transition between these two areas creates a region of the cortex which experiences very low peak strain magnitudes. Even though this neutral axis is far removed from the area of the cortex subject to the peak strains, somehow tissue is retained in this low magnitude strain

state. A conceivable mechanism to save bone from resorbing in this region could be differential coupling of bone cells to the matrix with cells in low strain regions being tightly coupled and cells from peak strain regions being more loosely coupled to the matrix. In this way, the cells have “tuned” themselves to the mechanical strain environment, a means of functional adaptation at the level of the cell. Certainly, bone cannot be presumed to be solely a compressive element, and strain cannot be presumed to be uniform across the cortex.

Recognizing that bone is first a tissue and second a mechanical structure, it is important to consider the biologic implications associated with physical stimuli. Indeed, tissue viability may depend on aspects of the mechanical environment which may not be at all rooted in maximal strain events. Alternatively, bone adaptation may depend on some camouflaged subset of the mechanical milieu, for example, the mechanical strains induced by muscle. While the symbiotic relationship between muscle and bone is inherently obvious, seldom is it explicitly considered in the context of one defining the other (50). As muscle contraction imposes far smaller strains on the skeleton than that caused by ground reaction loads (e.g., impact), their role in defining bone morphology has not received much consideration. Though muscle induced strains may indeed be relatively small, they are sustained for extended periods of time (e.g., in postural muscle activity), and thus—over time—may dominate the “strain history” that a bone is exposed to.

Examining this hypothesis, data from a variety of animals reveal the existence of a broad range of strains over a wide range of frequencies in the appendicular skeleton, even during activities such as quiet standing (51). In a variety of animals studied, an event of 1000 $\mu\epsilon$ occurred approximately once a day, while a 100 $\mu\epsilon$ event was 100 times more frequent, and events on the order of a few microstrain occurred tens of thousands times a day (Fig. 4). While reaction forces due to locomotion give rise to the large distinct strain events (1000 $\mu\epsilon$) at low frequencies (<5 Hz), the extremely small strain events are engendered at high frequencies with significant strain information extending out to the 20 Hz range. Whether the skeleton is preferentially sensitive to a few large strain events or a continual barrage of low magnitude events must be evaluated at the tissue level, where specific mechanical signals can be introduced and the resultant remodeling evaluated.

In summary, it is clear that the skeletal organ is subject to a wide range of mechanical signals, from low to high frequency strains, normal and shear strains, compressive and tensile strains. It is also clear that the cells on and within the mineralized matrix are subject not only to mechanical parameters such as strain, but derivatives of tissue deformation such as fluid flow and electrokinetic currents, parameters which may represent an important physiologic pathway in mediating an adaptive response. This accentuates the importance of studying the adaptation of bone to mechanical stimuli at its tissue level.

WHAT MECHANICAL PARAMETERS INFLUENCE BONE ADAPTATION?

The design of exercise protocols that can effectively stimulate bone growth or inhibit resorption requires the identification of specific mechanical parameters that can achieve this goal. For instance, if strain magnitude was identified as the single most important parameter, then large loads should be imposed. If, on the other hand, strain rate is more important, then exercise interventions should apply loads (possibly at low magnitudes) very rapidly. Similarly, determining that only a few cycles are necessary to saturate the response may allow interventions that are of minimal duration. Or, if a change in the distribution of bone strain (as defined by bending) is required, then complex exercises

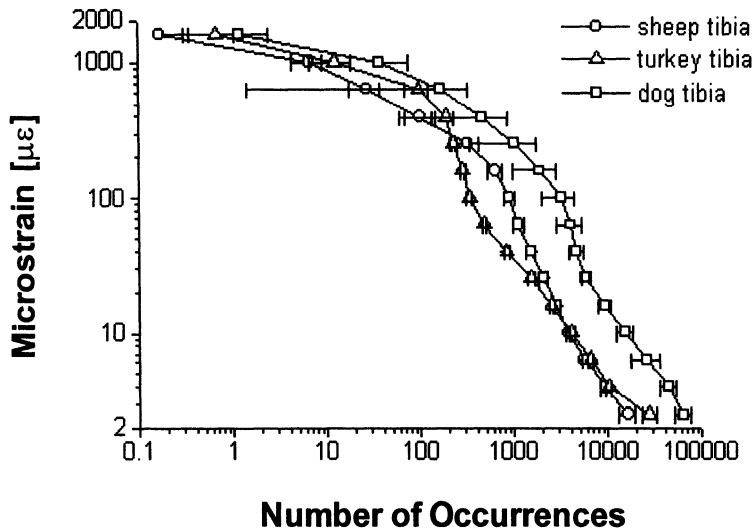


Fig. 4. Strain events measured in a sheep, turkey, and dog tibia over a 24-h period. Large strain events approaching $2000\mu\epsilon$ were exceedingly rare and the total number of strain events occurring during the 24-h period (strain history) were dominated by strain events that were very small in magnitude ($2\text{--}10\mu\epsilon$) but high in frequency ($>5\text{ Hz}$). Thus, the strain magnitude of an event decreased with its number of occurrences. Reprinted with permission from ref. 51.

creating diverse loading conditions would be called for. Relating specific aspects of bone's mechanical milieu to an adaptive response is a critical step towards efficient biomechanical interventions and this consideration has led to the development of models in which the applied mechanical milieu can be quantified at the tissue level.

Models that have been used to investigate bone's adaptive response to its mechanical environment include overloads induced by osteotomies (52), vigorous exercise (49), or exogenous loading in which external forces are applied to the bone. Physical exercise represents a physiological means of enhanced mechanical loading, but a disadvantage of exercise models is the limited exercise repertoire of most laboratory animals that makes it difficult to generate and control distinct mechanical milieus. Exogenous loading models such as the functionally isolated avian ulna (53), the axially loaded rat ulna (54), or the rat tibia placed in a four-point bending apparatus (55) allow the generation of controllable mechanical environments but a disadvantage of some these models is that the morphological response may be confounded by injury caused by the means of load application (54).

Since Wolff's treatise (Wolff's Law) (3), (physical laws govern the modeling and remodeling processes in bone) attention has focused primarily on strain magnitude as the dominant determinant of bone mass and morphology. Other parameters have received limited attention. Isolating the effect of a single mechanical parameter is not trivial because of the interdependence of many of the parameters. Changing strain magnitude while maintaining a constant loading frequency, for example, may result in a concomitant change in strain rate (Fig. 5). Despite these difficulties, several mechanical parameters have emerged from controlled studies that relate the mechanical environment to induced morphological changes.

Strain Magnitude

When holding strain frequency and number of loading cycles constant, longitudinal normal strain magnitude (strain in the direction of bone's longitudinal axis) is highly

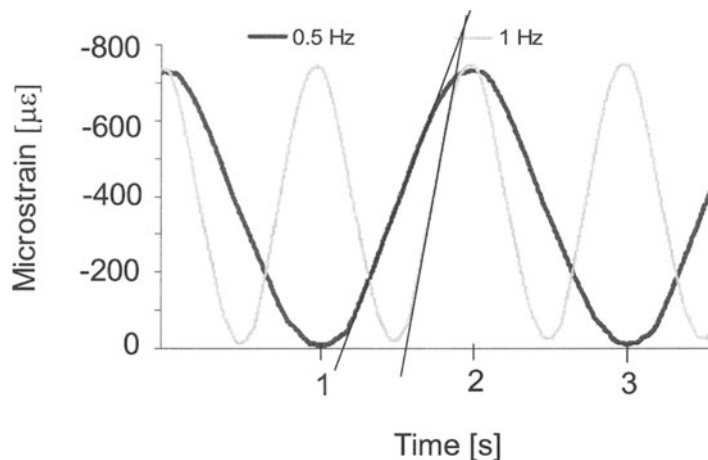


Fig. 5. Recording from a strain gage attached to the ventral surface of the turkey ulna subjected to either a 0.5- or a 1-Hz load. Both frequencies cause longitudinal normal strains of about 700 $\mu\epsilon$ in compression at this strain gage, but strain rate, the change in strain magnitude over time, is affected by the altered frequency. The 1-Hz signals induces peak strain rates that are twice as large (2200 $\mu\epsilon/s$) as the peak strain rates induced by the 0.5-Hz signal (1100 $\mu\epsilon/s$) as shown by the different tangents of the sine waves.

related to the osteogenic response (54,56,57). In other words, the larger the maximal deformation that is generated in the bone the larger the overall response of the bone. Strains below a certain threshold cause bone loss. This relationship was first demonstrated in the functionally isolated turkey ulna preparation to which strains in the range of 500–4000 $\mu\epsilon$ were applied for 100 cycles/d. In this model, the ulna of adult male turkeys is functionally isolated by proximal and distal epiphyseal osteotomies, leaving the entire diaphyseal shaft undisturbed. The only stimuli applied to the diaphysis are the mechanical regimen prescribed by the investigators, with no aberrant biophysical signals entering the preparation. In this model, strains smaller than 1000 $\mu\epsilon$ caused bone loss while strains larger than 1000 $\mu\epsilon$ lead to new bone formation in a dose dependent manner (Fig. 6A).

How much strain must be generated to obtain an osteogenic effect is dependent upon the interrelationship between strain magnitude, strain rate, and strain frequency. While in the previously described isolated turkey ulna preparation 100 loading cycles per day at 1 Hz inducing 1000 $\mu\epsilon$ prevented bone loss from occurring, this threshold can be reduced to 700 $\mu\epsilon$ when 600 loading cycles are applied at 1 Hz (58), to 270 $\mu\epsilon$ when 36,000 loading cycles are applied at 60 Hz (58), or to 100 $\mu\epsilon$ when 108,000 loading cycles are applied at 30 Hz (Fig. 6B) (70). This demonstrates that the search for a particular strain (loading) threshold has to take other mechanical parameters into account, and that this relationship can be exploited to design safer exercise regimens using smaller loads.

The complete strain state of bone tissue (strain tensor) induced by a functional regimen is very complex but can be described in general terms by two predominant components, normal strains and shear strains. While only normal strain were considered in previous studies investigating the relation between strain and new bone formation, it is essential to know whether normal, shear or some combination strain actually influences the metabolism of the osteocyte, osteoblast, or osteoclast to retain bone homeostasis, initiate modeling, or turn on remodeling.

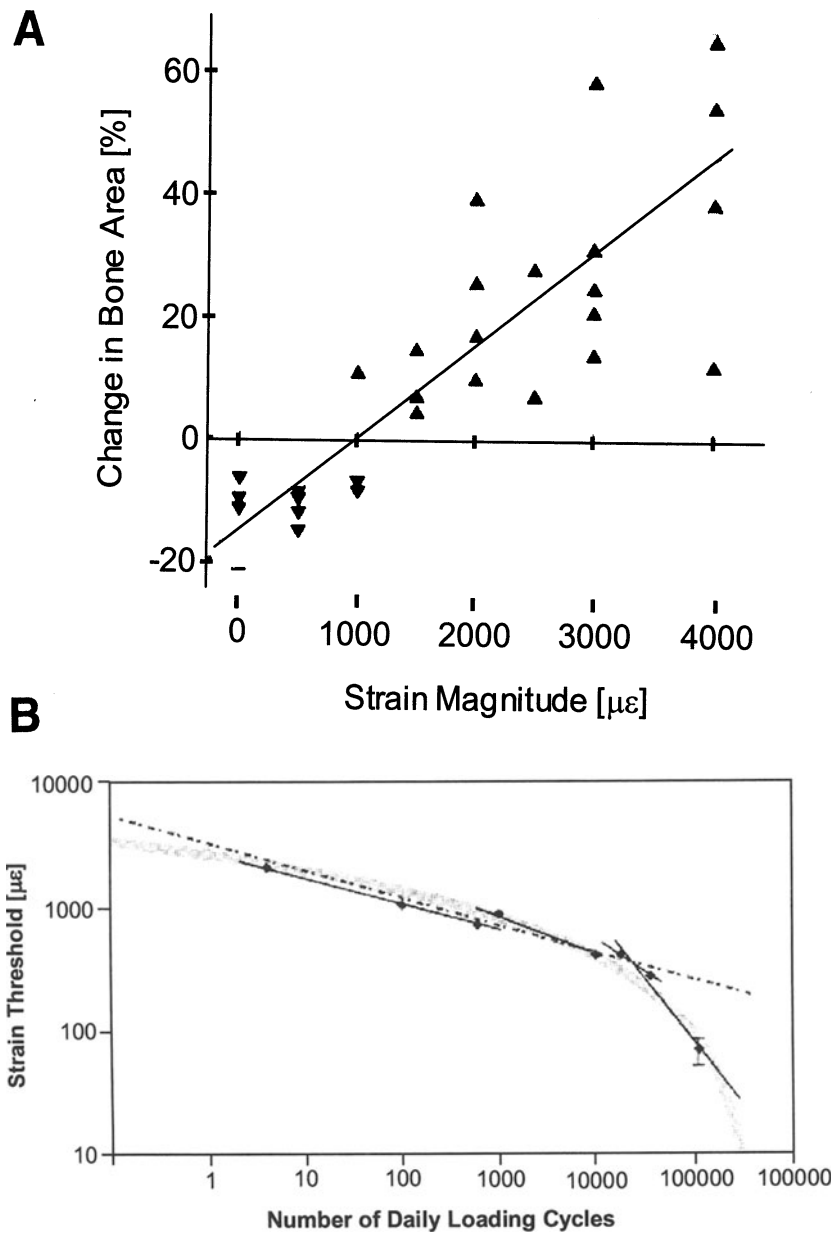


Fig. 6. (A) Relation between strain magnitude and changes in bone morphology when 100 cycles per day were applied at 1 Hz. Peak strain magnitudes engendered in the diaphyseal turkey ulna of about 1000 $\mu\epsilon$ retained bone mass. Strain magnitudes below this threshold caused bone resorption and strain magnitudes above 1000 $\mu\epsilon$ were osteogenic in a dose dependent relationship. Adapted from Rubin and Lanyon (57). (B) Empirical evidence compiled from several studies suggesting that the strain threshold at which bone mass is maintained (grey line) can be lowered when the number of daily loading cycles is increased; either 4 cycles of 2000 $\mu\epsilon$ at 0.5 Hz, 100 cycles of 1000 $\mu\epsilon$ at 1 Hz, or 108,000 cycles of 100 $\mu\epsilon$ at 30 Hz provide the same information with respect to the maintenance of bone tissue. Adapted from ref. 70.

This goal of investigating the osteogenic effects of different aspects of the strain tensor has been approached using the turkey ulna model of disuse osteopenia, in which

the modeling and remodeling response was quantified following 4 wk of either axial or torsional loading or disuse (59). Each of the two load groups were subject to peak principal strains of 1000 $\mu\epsilon$ (predominately normal strain in the axial case, and shear strain when subject to torsion). Of the three distinct groups, only disuse caused a significant change in gross areal properties (13% loss of bone). This suggests that both axial and torsional loading conditions are substitutes for the functional signals normally responsible for retention of bone mass, leaving the periosteal and endosteal envelopes unphased by disparate components of the strain tensor.

The intracortical response, however, was found to depend strongly on the manner in which the bone was loaded. Disuse failed to increase the number of sites within the cortex actively involved in bone turnover (intracortical events), yet area was lost within the cortex due to a threefold increase in the mean size of each intracortical remodeling site. Axial loading increased the degree of intracortical turnover as compared to intact controls, yet the average size of each remodeling event remained identical to that of control. Conversely, torsion elevated neither the number of remodeling events nor the area of bone lost from within the cortex, nor the size of the remodeling event. It appears that bone tissue can readily differentiate between distinct components of the strain tensor, with strain *per se* necessary to retain coupled formation and resorption; shear strain achieving this goal by maintaining the status quo, while normal strain elevates intracortical turnover but retains coupling. These data suggest that different components of the strain tensor have distinct regulatory roles. It is not the aggregate of strain *per se* that defines remodeling, but independent components of the strain tensor have differential responsibilities in achieving and maintaining bone mass.

Strain Rate

While strain magnitude appears to be an important determinant of bone mass, it is critical to realize that dynamic but not static strains have osteogenic potential. At the extreme, static loading (strain rate = 0) at strain magnitudes capable of stimulating formation when applied dynamically, produces a remodeling response similar to disuse that results in bone resorption (60,61). Several studies support the notion that bone is sensitive to the applied strain rate, with higher strain rates being more osteogenic (49,62–64).

For instance, high speed running (1.7 m/s) increases peak strain magnitudes by approx 20% in the middiaphyseal tarsometatarsus of roosters compared to walking (0.5 m/s) (65). This increase in strain magnitude is not accompanied by additional bone formation in the tarsometatarsal middiaphysis when growing roosters were exercised for 15 min/d (~2600 loading cycles) for 8 wk. An exercise intervention designed to employ high impact drop jumps revealed different effects (39). Middiaphyseal tarsometatarsal peak strain magnitudes induced by impact were similar to those induced by high speed running but strain rates were increased by 260% (0.32 e/s vs 0.09 e/s). In contrast to the running protocol, two-hundred drop jumps per day for 3 wk significantly increased bone formation rates at periosteal (+40%) and endocortical surfaces (+370%). The differential osteogenic effect associated with these two exercise protocols could be attributed directly to the large difference in generated strain rates. Site-specific analyses within the middiaphyseal cortex revealed that drop-jumping deposited additional bone preferentially in those regions that were subjected to the largest strain rates. This further emphasized bone's sensitivity to high strain rates.

When considering the ideal design of exercise interventions, these results imply that loads should be applied rapidly. Although exercise studies have been unable to identify

a specific exercise intervention that is most effective in producing beneficial skeletal effects, a trend has emerged that suggests high-impact exercise is more efficient than low impact exercises in stimulating new bone formation. This trend may support the notion that high strain rates have a critical impact on bone morphology. Unfortunately, bone strain rates have rarely been measured in humans and the occurrence of high strain rates in presumably high impact sports such as gymnastics or volleyball still has to be verified.

Cycle Number

A threshold behavior exists for the number of loading cycles. The full response can be triggered after only a limited number of loading cycles (53,66). In the functionally isolated turkey ulna preparation, a loading regime inducing peak strains of approx 2000 $\mu\epsilon$ maintained bone mass with only four cycles a day. When the cycle number was increased, this particular loading regime stimulated new bone formation. Thirty six load cycles saturated the osteogenic response, with as many as 1800 cycles being not more effective than 36 cycles (53). The notion that a finite number of loading cycles employing large loads may increase BMD or inhibit bone loss is supported by exercise studies involving weight lifters (7,15). It is critical to realize, though, that the saturation threshold of cycle number is influenced by other mechanical parameters including strain magnitude (as described above).

Strain Distribution

While a relation between peak strain magnitude and the resulting adaptive response has been proposed, bone also appears to be sensitive to how the strains are distributed across a section. Simply imposing a strain distribution that produces similar peak strain magnitudes as habitual loading conditions—but at different locations within the section (i.e., rotating the strain distribution)—may initiate new bone formation (67). Thus, unusual strain events (strain errors) have been suggested to drive bone adaptation. Running, for instance, may not be the osteogenically optimal exercise partly because it may generate strain distributions that are very similar to strain distributions induced by normal walking (Fig. 4) (40). Interestingly, sports that involve a great variety of changes in loading directions (such as soccer or badminton) have been suggested to possess a higher osteogenic capacity. However, it has not been confirmed that these changes in loading directions actually cause altered bone strain distributions.

Strain Gradients

Parameters such as peak strain magnitude or strain rate were primarily tested at the organ level. In other words, the region of the bone that was studied in response to a given mechanical stimulus was large (e.g., the middiaphysis of a long bone) and encompassed a large range of strain magnitudes. For instance, applying peak strain magnitudes of 2500 $\mu\epsilon$ in bending may produce 2500 $\mu\epsilon$ in compression on one side of the middiaphyseal cortex, 2000 $\mu\epsilon$ in tension on the other cortex and very low strains at the midline between these cortices. Rather than simply considering the peak magnitude of the stimulus and averaging the morphological response across a section, one could investigate whether new bone is actually deposited in those regions where the applied stimulus is the largest (i.e., the distribution of a mechanical parameter can be correlated with the distribution of bone's response). If such a site-specific relationship exists, then the knowledge of this specific osteogenic component may provide information about a mechanism by which

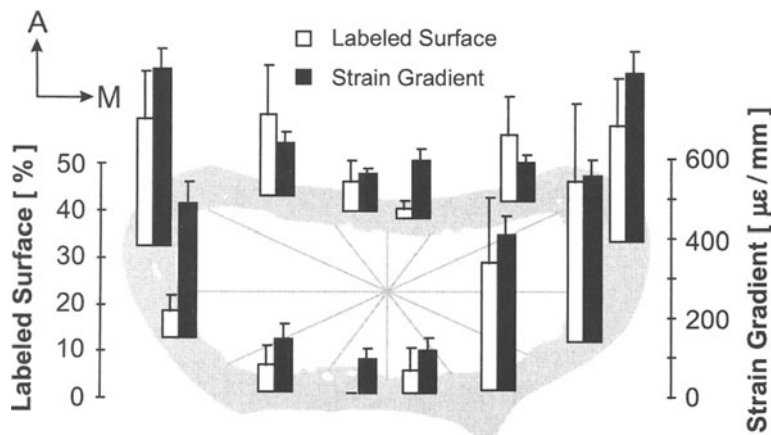


Fig. 7. Distribution of circumferential strain gradients and percentage of periosteal labeled surface per sector superimposed on a middiaphyseal TMT cross-section (Mean + SE). Sectors with the largest strain gradients correlated highly ($R^2 = 0.63$) with the sectors that exhibited the largest regions of bone forming surfaces (mean \pm SE). Reprinted with permission from ref. 30.

bone cells perceive their mechanical environment. Further, if a particular mechanical parameter is capable of consistently predicting the specific sites of bone formation in different models, then exercise interventions could be designed to deposit bone at sites where additional structural strength is required.

This issue was addressed in an exercise study in which young adult rooster ran on a treadmill for 9 min/d (~ 1500 gait cycles) for 3 wk (30). Strain gages were attached to the tarsometatarsus to determine the distribution of candidate mechanical parameters across a middiaphyseal section. Periosteal activation (as measured by histomorphometry) as well as mechanical parameters (such as strain magnitude, strain rate, and strain gradients) were quantified in twelve thirty degree sectors of a transverse section, thus enabling a site-specific correlation with each other (Fig. 7). The brief daily running regime activated periosteal surfaces but the amount of periosteal mineralizing surfaces per sector was only weakly associated with strain magnitude ($R^2 = 0.24$, negative correlation). In contrast, circumferential strain gradients (changes in strain magnitude across a volume of tissue) correlated strongly ($R^2 = 0.63$) with the sites of periosteal activation (Fig. 7), confirming earlier results from an external loading model (68). Generally, circumferential strain gradients are largest where strains (deformations) are the smallest. While it is counterintuitive from a structural (engineering) perspective that new bone formation is activated at sites subjected to low strains rather than large strains, physiologically it is important to point out that strain gradients are proportional to fluid flow in bone (69), a byproduct of strain which has been implicated to play an important role in mechanotransduction in bone. The finding that the distribution of strain magnitude was poorly related to the specific sites of bone formation may also imply that bone is possibly not sensitive to strain magnitude per se but rather to a parameter or combination of parameters that is only indirectly related to strain magnitude (such as strain gradients).

Further, as exercise-related new bone accretion is linked to specific sites within a bone, the structural enhancement of bone could be maximized if the deposition of new tissue could be directed towards sites that are at greatest risk to fracture. If, strain gra-

dients can be confirmed as consistent predictors of the specific sites of bone formation, then exercise protocols could be designed in which the sites of very low strains (large circumferential gradients) are aligned with those sites within the bone that would most efficiently enhance the structural strength of the tissue.

Strain Frequency

A common theme of the mechanical parameters described above is that only peak events are considered (e.g., peak strain magnitude, peak strain rate). From this, one could conclude that mechanical modulation of bone physiology depends on large signals to have any morphologic impact. However, the weak correlation of new bone formation with exercise intensity or with the specific sites of peak strain magnitudes, suggests that other factors may also be relevant for defining bone mass and morphology.

As indicated above, a nonlinear interdependence between cycle number, strain frequency, and strain magnitude was observed in the functionally isolated turkey ulna preparation (70). When loaded at 1 Hz, peak strains larger than 700 $\mu\epsilon$ are necessary to maintain bone mass. This loading threshold can be reduced to 70 $\mu\epsilon$ at 30 Hz (70). The ability to reduce this strain threshold is most likely influenced by the increased frequency at which loading occurred. The concept that high frequency but low magnitude strains can be osteogenic is supported by previous studies demonstrating that whole body vibration at 1 g (1 g = Earth's gravitational field) of young rats at 25 Hz for 12 h/d up to 120 d increased the modulus of elasticity and the microhardness of cortical bone harvested from the femur (71). Even lower levels of vibration (0.4 g) applied to the chest of the infant human, for purposes of mobilizing bronchial secretions, promoted the formation of radiologically evident periosteal new bone with just six 5-min treatments per day (72).

Since high frequency but low magnitude mechanical stimuli can stimulate bone formation, it is important to consider whether their presence is also responsible for preventing bone resorption. This hypothesis is based on *in vivo* strain data that indicate the presence of small magnitude but high frequency strains during functional activities. Animal models for bone resorption (such as rat-tail suspension) would remove this low level regulatory stimulus from the hindlimbs, and may therefore allow for resorptive activity and less bone formation. Strain as a stimulus to prevent osteoclastogenesis has been demonstrated both *in vitro* (73) and *in vivo* (53) but these observations were based on large magnitude strains at low frequencies (0.5–5 Hz).

Female, 6-mo-old Sprague-Dawley rats were used to test whether the exposure of bone to high frequency but low magnitude stimuli may prevent bone resorption (73a). First, the extent to which whether tail suspension (hindlimb disuse) induced changes in bone formation rates was assessed. It was then determined whether a mechanical signal consisting of high frequency whole body vibration at 90 Hz (0.25 g) for 10 min/d can return altered bone formation rates to normal in hindlimb suspended animals. The ability of this high frequency mechanical stimulation to rescue the bone phenotype was compared to control rats, as well as to rats subjected to tail suspension interrupted by weight bearing for 10 min/d. At the end of the 28 d protocol, we found that hindlimb suspension decreased trabecular bone formation rates by 92% compared to controls. Hindlimb suspension interrupted by 10 min of weight-bearing per day failed to negate bone loss. In contrast, when high-frequency low-magnitude mechanical stimulation was used for 10 min/d to combat disuse, the impact of the intervention served to normalize the re-

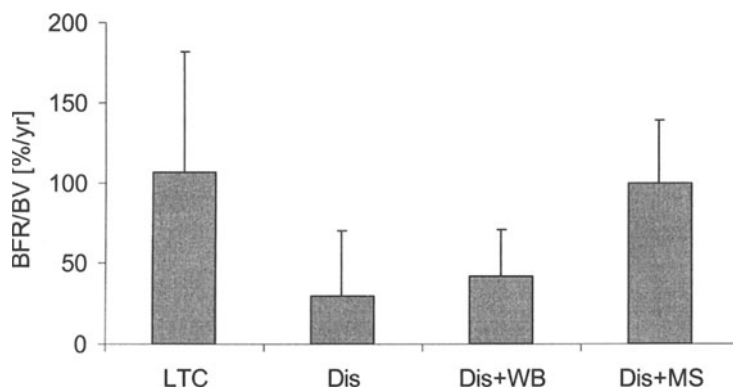


Fig. 8. Tibial trabecular bone formation rates (BFR/BV) of age matched controls (LTC) and after 28 d of hindlimb suspension related disuse (Dis), disuse interrupted by 10 min/d of weightbearing (Dis+WB), and disuse interrupted by 10 min/d of high frequency low magnitude mechanical stimulation (mean \pm SD). Adapted from ref. 73a.

sponse back to control values (Fig. 8). This demonstrates the capability of extremely low level vibration to inhibit the decline in bone formation rates associated with hindlimb disuse. Clinically, these data are encouraging since this safe biomechanical intervention effectively prevented disuse related osteopenia from occurring, even when the bone was subjected to 23 h and 50 min/d of this strong stimulus for resorption. Based on these observations, high frequency, low magnitude, mechanical strains may serve as a “surrogate” for musculoskeletal forces and, thus, may represent a countermeasure to the osteopenia which parallels disuse.

This “other than peak” perspective is employed in several biologic systems which perceive and respond to exogenous stimuli, such as vision, hearing, and touch. In considering the mechanically mediated control of bone remodeling, there is little argument that biophysical stimuli are potent determinants of skeletal morphology but excess strain may only damage the system. To identify the criteria by which these processes are controlled, it is necessary to look beyond the material consequences of a structure subjected to load and consider the biologic benefit of a viable tissue subjected to functional levels of strain.

Summary

This brief review of the effects of specific mechanical parameters on skeletal adaptation demonstrates the complexity of the interactions between the produced mechanical environment and bone’s response to loading. The variety of bone adaptation phenomena cannot be successfully explained for by a single mechanical parameter. It is likely that bone is responsive to a combination of parameters and their individual importance may depend on a variety of factors such as age and hormonal status. It is also conceivable that bone is only indirectly related to mechanical matrix strain and that bone cells sense mechanical stimuli applied to the bone through a factor that is a byproduct of matrix strain such as interstitial fluid flow, in turn stimulating bone cells through fluid shear stresses or electrokinetic effects. Fluid flow in bone is proportional to pressure gradients generated in bone, which themselves are highly related to strain rate as well as strain gradients perhaps accentuating the physiological importance of these two parameters.

EXAMPLE FOR A SAFE CLINICAL APPLICATION OF BIOPHYSICAL STIMULI

Transmission of Low Level Mechanical Stimuli to the Skeleton

While a biophysical approach contrasts sharply with pharmaceutical strategies for the treatment of osteoporosis, in essence the structural success of the skeleton is a product of its ability to adapt to a constant barrage of mechanically based signals. As outlined earlier, animal work has led us to the hypothesis that high frequency but low magnitude mechanical stimuli can influence bone formation and resorption. Since these signals are so small, they could easily be used clinically to inhibit or reverse osteopenia. Certainly, low-level biophysical signals would be simpler and safer to impose than large stimuli that are typically associated with exercise regimens.

As a first step, the feasibility of transmitting low level mechanical stimuli to the human skeleton was examined. The nature of the weight-supporting skeleton facilitates the transmission of mechanical energy into bone tissue in a relatively direct manner. Because the weight-bearing skeleton certainly subjects the skeleton to strain, a dynamic strain on the skeleton can presumably be induced by perturbing its effective gravity. The modulation of g-force can be accomplished by placing the standing human on a platform which is made to oscillate at a specific frequency and acceleration (74). The strains arising from dynamic alterations in g-force would be transferred into the skeleton along a normal trajectory, ensuring that the stimulus is concentrated at those sites with greatest weight bearing responsibility (e.g., femoral neck), yet weak at sites not subject to resisting gravity (e.g., cranium). While conceptually simple, it must be demonstrated that ground based accelerations are indeed transmitted through the bones and joints of the lower appendicular skeleton; little is known of transmissibility of ground based vibration at frequencies above 12 Hz (75). To establish the relationship between acceleration at the plate surface and transmission of acceleration through the appendicular and axial skeleton, accelerations were measured from the femur and spine of the human standing on a vibrating platform. Force transmission to these bones was determined using accelerometers mounted on Steinman pins transcutaneously imbedded in the spinous process of L3 and the greater trochanter of the right femur of six volunteers (74). To determine damping as a function of posture, data were also collected from subjects while standing with bent knees.

Negligible loss of acceleration was observed in the femur and spine up to frequencies of 30 Hz but transmissibility fell off by as much as 60% when the frequency approached 40 Hz. Further, when the subject was asked to stand with bent knees, transmissibility fell to below 20% at the femur and spine. Presumably, this is due—at least in part—to the uncoupling of the body segments, such that they are no longer working efficiently as a fixed, stiff system. More importantly, these measurements confirm the ability of the standing adult skeleton to transmit a substantial fraction of ground accelerations to regions of the weight-bearing skeleton most susceptible to bone loss.

Potential of Low Level Mechanical Stimuli to Prevent Postmenopausal Bone Loss

The design and development of a prototype device suitable for humans led to a 1-yr feasibility trial on a small cohort of women in which we evaluated the ability of a low magnitude (0.2 g), high frequency (30 Hz) mechanical stimulation to inhibit postmeno-

pausal bone loss in a prospective, randomized, double blind, placebo-controlled clinical trial (76). Sixty-two women, 3–8 yr postmenopausal, enrolled in the pilot study. Thirty-one women underwent mechanical loading of the lower appendicular and axial skeleton for two 10-min periods per day, induced via floor-mounted devices that produced the mechanical stimulus. Accelerations of 0.2 g are just perceptible and no adverse reactions were reported. Thirty-one women received placebo devices and underwent daily treatment for the same period of time. DXA was performed on the spine (L1–4), right and left proximal femur, and non-dominant radius at baseline and at approx 3, 6, and 12 mo. A full complement of DXA data was obtained for 56 of the patients (28 treatment, 28 placebo; six subjects dropped from the study for reasons not related to the device). In a *post hoc* analysis, a linear regression of the means was used to show that lumbar spine bone mineral density (BMD) declined by -3.3% (± 0.83 , $n = 28$) in the placebo group compared to only -0.8% (± 0.82 , $n = 28$) in the treated group ($p < 0.03$), reflecting a 2.5% benefit of the biomechanical intervention. A 3.3% treatment benefit was observed in the trochanter region of the hip, with a -2.9% (± 1.2) loss observed in the placebo group, yet with a 0.4% (± 1.2) gain in the treated group ($p < 0.03$). At the distal radius, no significant differences were observed as a function of time or between groups, indicating the biomechanical influence was localized.

Stratifying the results based on patient body mass index (BMI), end point analysis confirms the relationship between svelte stature and a greater degree of bone loss (77); placebo subjects with $\text{BMI} \leq 25$ lost 2.5% (± 0.6) BMD over the course of the year, while those with a $\text{BMI} > 25$ did not show any change over the 12-mo period. This stratification also demonstrates the ability of mechanical stimulation to inhibit this bone loss in the group at greatest risk; in subjects with $\text{BMI} \leq 25$ who were exposed to the mechanical stimulus the bone loss in the spine was not significantly different from zero ($+0.2\% \pm 0.7$). The 2.7% difference between placebo and treatment groups was significant at $p < 0.01$. Treated subjects with $\text{BMI} > 25$ showed no apparent effect of treatment, perhaps because there was no bone loss in placebo-treated subjects. Overall, these results indicate the potential of a unique, noninvasive biomechanical therapy for osteoporosis. It represents a nonpharmacologic means of inhibiting the rapid decline of bone mineral density which follows menopause.

SUMMARY AND OUTLOOK

The critical role that biophysical stimuli play in achieving and maintaining a structurally appropriate bone mass is clear. It is also clear that bone is capable of responding to exercise induced mechanical stimuli by improving or maintaining its quality and quantity. Although many exercise regimens have been ineffective in producing any morphological changes, bone cells should not be accused of being unresponsive to mechanical stimuli. Rather, exercise regimens will have to be designed that target specific skeletal sites with the right amount and components of the complex mechanical environment generated by exercise. To enable the formulation of these exercise interventions, the mechanisms by which bone senses mechanical stimuli have to be explored at the organ, tissue, and molecular level.

A trial and error approach to finding an osteogenically optimal exercise program is highly inefficient. Instead of attempting to associate specific physical activities to altered BMD, mechanical milieus induced by these activities should be described. Then their characteristics can be correlated with changes (of lack of them) in bone morphology. It

may also be time to develop and employ measurement techniques that are capable of detecting focal changes in bone mass and that can also distinguish changes in bone quality (e.g., morphology) from changes in bone quantity. At the same time, specific mechanical parameters derived from carefully controlled animal studies should be incorporated into the design of exercise protocols and tested. Current data from studies examining the ability of specific components of the mechanical milieu to stimulate bone formation indicate that osteogenic mechanical stimuli neither have to be large in magnitude nor do they have to be applied over a long duration, important prerequisites for the design of clinically successful exercise interventions. Ultimately, exercise protocols will also have to take the systemic state of the patient into account (e.g., stage of development, age, hormonal, and nutritional status), as it is unlikely that one universally applicable exercise regimen will provide maximal efficacy to the skeleton in all clinical situations.

In summary, exercise may represent a unique means to increase bone mass as well as to prevent bone loss. Since the strain generated by exercise is native to the bone tissue and incorporates all aspects of the remodeling cycle (an attribute that is not shared by systemic, pharmaceutical interventions) biomechanical interventions may provide unique advantages. Results from exercise interventions, however, have been ambiguous and the widespread use of exercise related mechanical stimuli in the treatment of skeletal disorders will be delayed until we achieve a better understanding of the mechanisms by which they act.

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11

Exercise in the Prevention of Osteoporosis-Related Fractures

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INTRODUCTION

The utility of exercise in preventing osteoporosis-related fractures is a topic of considerable interest and research effort. It is well-known that skeletal unloading, such as occurs following spinal cord injury, prolonged bed rest, limb immobilization, and microgravity, precipitate generalized skeletal loss, particularly in bones that bear weight under normal conditions (1–5), and these losses are not always entirely regained upon return to weight bearing in normal gravity. On the other hand, the association between loading from exercise and bone accretion is both highly variable and poorly understood. Skeletal responses to exercise vary strongly as a function of bone age, reproductive hormone status and experimental design, including the length of time of the intervention. In humans, the goal of an exercise intervention is not only to increase bone mineral density, but also to reduce fractures. The etiology of osteoporotic fractures includes both low bone density and falls. Falls account for over 90% of hip fractures and over 50% of vertebral fractures. Thus, osteoporosis-related fractures are both disease and injury and developing interventions that serve to improve bone density and to prevent falls will ultimately provide the most effective strategy to reduce fractures. This chapter provides a discussion of exercise as a means of reducing the factor of risk in order to prevent

Osteoporosis-related fractures and an overview of these elements of exercise. It also includes a discussion of study design. We summarize the literature specific to promoting bone health across the lifespan and discuss the importance of exercise as a strategy for fall prevention. Lastly, we provide our conclusions for future research and also exercise prescription for reducing the factor of risk.

FACTOR OF RISK

The factor of risk, based on engineering principles, is defined as the ratio between the applied load and the fracture load ($\phi = \text{applied load/fracture load}$). If the applied load is greater than the fracture load, then fracture is probable. If the applied load is less than the fracture load, fracture is unlikely. For example, in a 70-yr-old individual with an average hip bone density, the factor of risk ranges from 1.25 to 3.0 for a fall from standing height (6–8). Exercise is unique in that it can reduce the risk of fracture by altering both the numerator and denominator of the factor of risk. It can affect the numerator in two ways. First, eliminating falls, the numerator becomes zero and fracture is highly unlikely. Second, by improving lower extremity neuromuscular function, exercise can reduce the applied load by lowering the energy of a side fall. To raise the denominator, exercise can increase bone mineral density and reduce skeletal fragility, thus increase the force required to fracture.

EXERCISE STUDY DESIGN

Attention to study design and implementation is a key factor in experimental research. This section serves as an overview of factors to be considered when designing exercise interventions in order to promote carefully-developed research efforts and ensure the credibility of experimental outcomes.

Drinkwater (9) has emphasized the need to incorporate five principles into exercise study design: specificity, overload, reversibility, initial values, and diminishing returns. Although attention to these principles is common in the fields of muscle and cardiovascular research, their emergence in studies of bone is more recent. An exercise protocol should be designed to load the target bone, that is, be *specific* to the site measured. Additionally, an exercise must *overload* bone in order to stimulate it. Lack of attention to overload is a frequent problem in published intervention studies, and one that is difficult to overcome given the limited information regarding the quantitative relationship between specific activities and the loads imposed at given skeletal sites. In addition, unlike the muscle and cardiovascular systems, bone has a “lazy zone” within which exercise that may overload and stimulate adaptation in the muscle and cardiovascular systems does not sufficiently overload bone (10). For example, a progressive program of jogging with the goal of 3–5 miles/wk resulted in improvements in aerobic capacity after a 9-mo period, but did not improve hip bone density, this is because, in the population of young women studied, although the activity was sufficient to overload the cardiovascular system, it was not different enough from daily activity at the hip to result in significant improvements in bone. *Reversibility* refers to the reversal in bone response once a stimulus is removed. This response is documented in the mature adult skeleton (11–13), but not apparent in the growing skeleton. Recent, but limited data in children demonstrate that the growing skeleton retains the gains achieved from increased mechanical loading (14). Cross-sectional reports in adults support this hypothesis as individuals that exercised before or during puberty have significantly higher bone mass than those who were less active (15). *Initial*

values refers to the concept that responses from bone are greatest when beginning levels are lower than average. This effect is not often reported in the literature, and thus there are few data that corroborate the tenet. However, we recently found that in a group of premenopausal women engaged in impact plus resistance training, those with the lowest initial bone density at the hip demonstrated the greatest responses after 12 mo of training (Winters & Snow, unpublished data, 2000). In addition, folk dancing 3 h/wk did not produce increases in spine BMD in postmenopausal women with osteoporosis but had no effect on BMD at this site in postmenopausal women with normal bone mass (16). An apparent contradiction to this principle is that if the load is extremely high (i.e., greater than 10 body weights), skeletal increases are observed regardless of initial values (17). However, this response serves to exemplify the significance of load magnitude in osteogenesis. *Diminishing returns* means that once a given training level is achieved, further responses will be slow and of small magnitude. This principle has not been specifically evaluated in the bone literature likely due to the fact that bone responds slowly to overload and few have evaluated bone long enough or across enough time points to observe a plateau in the bone response. Consideration of these principles in research design will provide a basis on which to design effective exercise interventions.

Other important factors that distinguish the exercise response of the skeletal system from that of the muscular and cardiovascular systems are: 1) changes are small (1–5%), 2) the time required to elicit a measurable response is considerable, 3) a progressive overload is not always necessary, 4) older bone is less responsive than younger bone. Bone requires at least 6 months to initiate adaptation and complete one remodeling cycle whereas both the neuromuscular and cardiovascular systems typically respond to a training stimulus within 4–6 wk. It is increasingly clear that bone does not require a progressive overload in order to allow time for adaptation (18,19). In fact, if the individual neuromuscular system can withstand the load, it is advantageous to begin with high magnitude early in the intervention period given the relatively long response time of the skeletal system. If an exercise design incorporates the time required to reach sufficient load magnitude to stimulate osteogenesis, significant time will be spent in the “lazy zone,” leaving less time for skeletal exposure to the desired load. However, a progressive approach to increasing load magnitude and preparing the body for activity may be necessary depending upon the age and health status of the individual. For example, to prepare older, postmenopausal women for jumping activity, we began with lower extremity weighted vest exercise in order to strengthen the ankle, knee and hip joints (20). Lastly, bone age is emerging as a factor in the skeletal response to exercise. Not only does younger bone appear to respond better to a similar activity than older bone (19), but a younger individual can withstand higher load magnitudes than an older person. Thus, specific exercise for increasing bone or reducing bone loss in the elderly requires lower load magnitude and longer training periods (>12 mo). For example, we observed that participation in weighted vest exercise plus jumping did not lead to a bone response after 9 mo of training in postmenopausal women, but prevented 4.4% bone loss at the hip after 5 yr of participation (13).

THE EFFECTS OF EXERCISE ON BONE MASS

Given the strong relationship between DXA-derived bone mineral density, bone mineral content and failure load, increasing bone mass and decreased loss are important strategies for reducing fractures (7,21). Bone requires sufficient overload in order to increase its material and/or geometric properties, the mechanisms that underlie the adaptation of bone to mechanical loading have been discussed in Chapter 10.

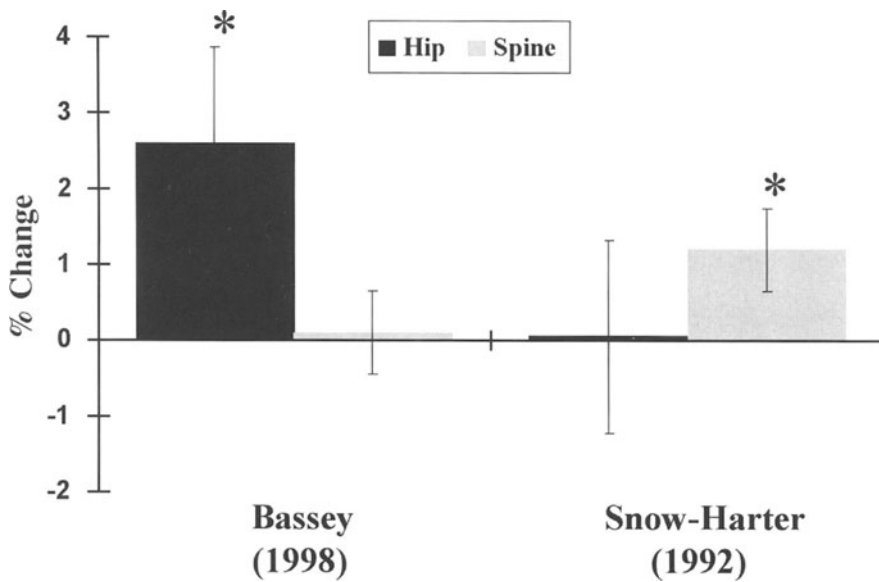


Fig. 1. Mean percentage change (\pm SEM) for between premenopausal exercisers and controls for randomized interventions utilizing impact exercise (jumping and running) and running to increase bone mass at the hip and spine. Bassey et al. (1998); significant increases in femoral trochanter BMD, and no differences in lumbar spine BMD after six months of jumping (50 jumps/day). Snow-Harter et al. (1992); significant increase in lumbar spine BMD, and no difference in femoral neck BMD after eight months of running (* $p < 0.05$).

The approach to developing an exercise prescription for stimulating the skeletal system requires defining both the type of exercise and then the optimal dose, that is, combination of load magnitude, loading rate, repetitions (load cycles) and frequency (sessions/wk). Type of exercise is important in that it must be site-specific and load the target bone, in most cases, the hip and/or the spine. Ideally, the approach to defining optimal overload should be undertaken in a manner similar to that of defining a prescription for drug therapy by carefully developing points on the response surface to relate bone mass increases to various combinations of dose, repetitions and frequency in order to develop the optimal combination of these variables.

“Dose” for bone includes load magnitude, the rate at which the load is applied, load cycles, and frequency of exercise (number of exercise sessions per week). Loading rate (peak force/time to peak force) appears to be of particular importance for augmenting hip bone mass in the young and mature adult skeleton as activities with higher loading rates (e.g., jumping) *consistently* increase hip bone mass whereas bone mass increases from activities with lower loading rates (e.g., aerobic activities such as running and resistance exercise) provide mixed results (22–26). While load magnitude is similar for jogging and jumping (2–4 times body weight), the loading rate for jumping can approach 200 body weights/s and for jogging only about 75 body weights/s. This difference may explain the fact that jumping activity increased hip trochanteric BMD 3% in mature premenopausal women (19,27), but jogging had no effect on hip bone mass in young premenopausal women (24) (Fig. 1). Loading rate increases when both load magnitude and the rate of force application increase.

The effect of load magnitude on bone is well-substantiated in both human and animal models (17,28–30). An example of the importance of load magnitude emerges from studies of muscle strength effects on bone. In cross-sectional studies, increased muscle mass and strength are associated with higher bone at both the hip and the spine. Additionally, exercise interventions using strength training have shown that high intensity strength (>80% of one repetition maximum) training produces increases in spine and hip bone mass (25) and is more effective than low and moderate intensity strength training (25,31).

The relative importance of rate versus magnitude is poorly understood. However, there are limited data in animals that indicate loading rate has an effect on bone that is independent of load magnitude (32,33). More investigation in this area will elucidate the independent and combined effects of these variables on bone mass accretion.

Although there is evidence from carefully controlled animal studies that load magnitude is more osteogenic than are loading cycles (17,28–30), the effect of varying load cycles on bone in humans is poorly understood. In a recent study of young children, we reduced load cycles from 300 jumps/wk to 150 jumps/wk and observed no response at the hip after 7 mo whereas 300 jumps had produced a 5% increase in hip bone mass over the same time period (Fuchs and Snow, unpublished data). More work in carefully-controlled trials will allow better understanding of the synergy between load magnitude, rate and load cycles in the human skeleton.

In summary, although a specific exercise prescription for increasing bone mass at the hip and spine is not defined, there is evidence that both load magnitude and loading rate are osteogenic and that rate is particularly effective at the hip. Furthermore, data are just emerging on the potential importance of the frequency content of the signals that overlay a load cycle (34). Continued efforts are necessary to better define safe and effective combinations of dose, repetitions and frequency from specific exercise activities for increasing hip and spine bone mass at important developmental stages across the lifespan.

EXERCISE STUDIES ACROSS THE LIFESPAN

In general, cross-sectional studies demonstrate that physically-active individuals have superior skeletal mass than those who are less active. The magnitude of this difference in bone depends upon the mode and intensity of the activity, the age at which it began, and the number of years spent in training. However, an important limitation of cross sectional studies is that of subject selection bias. That is, individuals who choose a specific type of exercise may have predisposing skeletal characteristics that influence this choice. For example, a higher bone density in a person who lifts weights may be due to the fact that their skeletons more easily support this activity rather than the actual effect of weight lifting on the skeleton. Another limitation of cross sectional studies is the use of standard activity questionnaires to evaluate physical activity. Most of these instruments are designed to measure energy expenditure and do not assess forces applied to the skeleton. Thus, attempts to relate bone mass to total energy expenditure include high degree of potential error and consequently, of inappropriate conclusions.

Exercise and Peak Bone Mass

In the recent NIH Consensus Conference on Osteoporosis, attaining higher peak bone mass is cited as a primary strategy in preventing osteoporosis. Thus, vigorous research efforts are underway to enhance bone accretion during growth.

CROSS-SECTIONAL REPORTS

Studies in children and adolescents of various races generally support significant associations between physical activity and total body, hip, spine, and forearm BMD (35–43). Evidence is accumulating to suggest that exercise confers the greatest long-term benefit when initiated in the prepubertal years (15,44). Prepubertal gymnasts have greater BMD at weight bearing sites than controls, an effect that strengthens as duration of training increases (38,39,45). Compared with less active children, highly active children have a greater rate of bone mineral accumulation for the 2 yr during which bone is most rapidly accruing (12.5 yr for girls and 14.1 yr for boys) (43). In their report, Bailey and coworkers noted that this greater accrual translated into 9 and 17% higher total body bone mineral content one year after peak bone mineral content velocity for active boys and girls, respectively. Kannus and others found that female tennis and squash players who began playing before menarche have twice as much bone in the humeral shaft as those who began playing after puberty (15). Slemenda and associates (46), however, found no relationship between physical activity and BMD in peripubertal children, and suggest that exercise exerts an influence on BMD before puberty, but that during puberty other factors, such as estrogen, become more influential on bone acquisition, than exposure to mechanical loading. By contrast, Haapasalo and colleagues (47) reported that the differences in spine BMD of athletic and control children were greatest at the peripubertal years Tanner stages IV and V (average ages [TS IV & V] 13.5 and 15.5, respectively). Variations in BMD response to different activities reflect the different loading patterns of each sport and the phenomenon of site specificity (42,48). The effect is clearly demonstrated by the fact that dominant limbs have greater BMD than non-dominant limbs (49), and athletes loading their dominant limbs preferentially while exercising develop even greater bilateral disparity (50,51). In summary, the data from cross-sectional studies agree that exercise confers the most positive benefits on the skeleton before puberty but provide mixed results as to the effect during the peripubertal years.

EXERCISE INTERVENTIONS

Since exercise interventions for bone have only recently targeted youth, there are few data in children. However, results are consistent with cross-sectional observations.

In a study of premature infants, five repetitions of range of motion, gentle compression, flexion and extension exercises five times a week resulted in greater acquisition of BMD at 4 wk in exercised babies than in controls (52). Six-month-old infants engaged in gross or fine motor activity for the next 18 mo exhibited enhanced bone accretion (53). In that study, calcium intake exerted a strong influence on the response with lower calcium associated with reduced accrual.

In a randomized study of 89 boys and girls (mean age = 7.1 yr), jumping 300 times/wk at ground reaction forces of eight times body weight increased femoral neck BMC and area 5.6 and 3.2%, respectively, over control children (14). This effect was maintained 7 mo after detraining (14a) indicating that the specific program enhanced growth in young children (Fig. 2). Areal BMD at the spine, legs and whole body increased in prepubescent boys (mean age 10.4 yr) compared to controls in response to eight months of weight bearing activity that supplemented regular physical education (54). A similar response was observed in young girls who performed resistance training plus jumping exercise for 10 mo (55). McKay et al. (56) used hopping and bounding activities and reported that trochanteric BMD increased 1.2% in prepubescent girls.

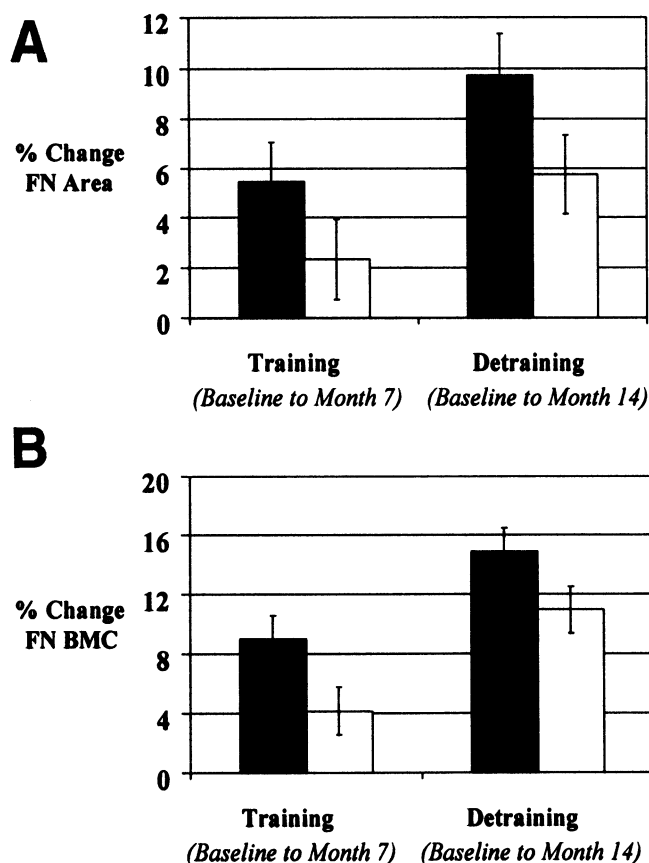


Fig. 2. Absolute values of femoral neck bone area (A) and bone mineral content (B) in a randomized exercise intervention of children who completed 7 mo of training followed by 7 mo of detraining. Repeated measures analysis of covariance (covariates: initial age, and height and weight change scores) identified a significant group by time interaction for femoral neck bone mineral content and bone area over 14 mo ($p < 0.01$, $p < 0.01$, respectively). After training, jumpers (black bar) were higher than controls (white bar) for bone mineral content and area, ($p < 0.001$, and $p < 0.01$, respectively). The higher values for femoral neck bone mineral content and area were retained after detraining ($p < 0.05$ and $p < 0.001$, respectively). Training data reprinted with permission from ref. 14.

To our knowledge, there is only one exercise intervention reported in adolescents. Following 8 mo of plyometric and jumping exercise in an adolescent female cohort (age = 14.2 yr), no significant difference between groups was observed at any bone site. However, trochanteric bone mineral content increased significantly in the exercisers, whereas there was no change at this site in controls (57). Because this trial was not randomized and controls were highly active, it is unclear whether the nonsignificant difference between groups was due to the high level of activity in controls or the fact that adolescent bone does not respond as dramatically to increased loading as does prepubertal bone.

Exercise and Bone Mass in Adults

Although, the response of the adult skeleton to exercise has been studied extensively, the difficulties of subject recruitment, randomization and compliance associated with exercise intervention trials have produced data that are inconsistent. Randomization is

particularly difficult since adults who volunteer for an exercise study do not wish to be controls and thus are commonly unwilling to be randomized. Additionally, there are few studies in men due to the frequent perception that osteoporosis is a disease in women.

CROSS-SECTIONAL REPORTS

Adults engaged in weight-bearing exercise at intensities of >60% of aerobic capacity have consistently greater BMD than nonexercisers or those exercising at low aerobic intensities. These differences have been observed in the whole body (58–66), spine, and/or proximal femur (37,58–61,63,65–74), pelvis (60,64), distal femur (75), tibia (58,60,72,76,77), humerus (60), calcaneus (78,79), and forearm (72). Broadband ultrasound attenuation and speed of sound transmission in the calcaneus are similarly higher in runners than controls (63). As expected given the principle of site specificity, the high BMD of athletes is observed at the skeletal sites loaded during their respective activities (64,80–82). Another example of this principle is the higher femoral BMD observed in individuals that engage in weight bearing activity in the workplace compared to those with more sedentary occupations (83).

Certain activities may not apply a sufficient stimulus to the skeleton to cause an adaptive response (84), thus the loading from these activities would be within the “lazy zone” previously described. Athletes participating in moderate to high intensity impact activities such as running, jumping and power lifting have greater bone mass than those performing low intensity or nonweight bearing activities (62,74,85,86). Individuals who participate in nonweight bearing activities such as swimming have similar BMD to nonexercisers (30,75), although limited data to the contrary exist in men (87). The degree of swimming participation may strongly influence the effect of the activity on bone density. Elite swimmers unload their skeletons by spending more than 20 h/wk in a nonweight-bearing activity. Muscle forces on the skeleton during swimming do not appear to offset the substantially reduced weight bearing activity in these athletes.

In nonexercising adults, as in children, the dominant arm exhibits greater total and cortical bone mass than the non-dominant arm (49,88) and greater differences between right and left side limb bone masses are evident when the dominant limb is chronically overloaded (50,51,68,75). The difference may be accounted for by increased periosteal area and cortical thickness rather than BMD (88). This dominance is not evident in rowers and triathletes (89), since the loading patterns from these activities are essentially equal bilaterally.

Although there is question as to the role of exercise in preventing age-related bone loss (90), active people who have exercised for many years generally have higher BMD than those who have been less active (39,61,67,91–94). Furthermore, although bone loss may be reduced by lifetime exercise, that benefit may have little or no relationship to fracture risk. For example, Greendale and colleagues (94) reported a significant linear trend in older men between both lifetime and current exercise and hip BMD, but found no relationship between osteoporotic fracture rate and exercise profile.

EXERCISE INTERVENTIONS IN YOUNG AND MATURE PREMENOPAUSAL WOMEN

Exercise training programs enhance the bone density of young women in a site specific manner. Both resistance and weight bearing endurance exercise programs increase spine, hip and calcaneal BMD of young adult women (18,19,24,95–97). Impact loading using jumping activities consistently results in increased trochanteric BMD of 2.5–4% in mature premenopausal women (12,18,19). However, in contrast to the developing

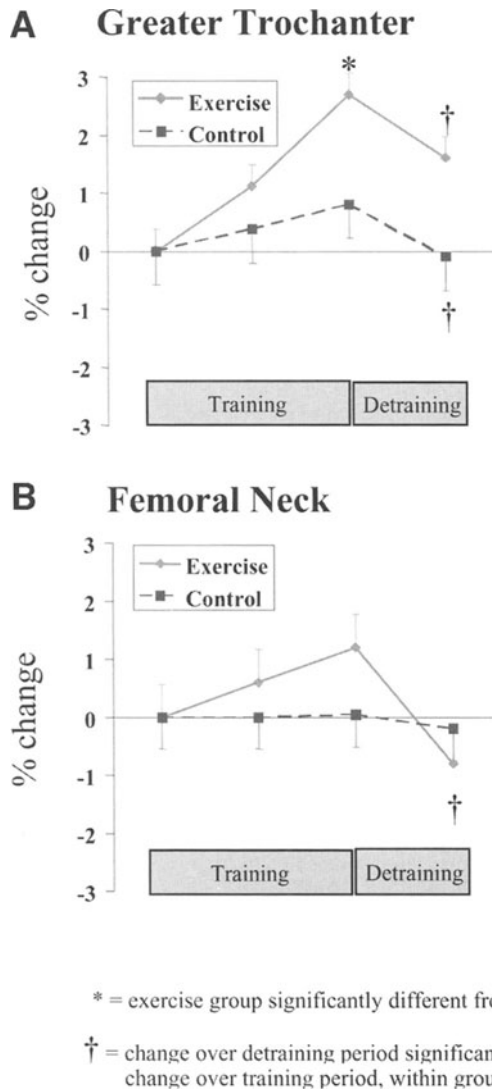


Fig. 3. Percent changes in bone mineral density in mature premenopausal women across 12 mo of resistance plus jump training and 6 mo of detraining. (mean \pm SEM) at the A) greater trochanter and B) femoral neck. Reprinted with permission, Winters & Snow, JBMR, 2000.

skeleton increased loading must be continued in order to maintain bone gains. In a recent report, Winters and Snow (12) observed that the increases in trochanteric and femoral neck BMD after 12 mo of resistance plus jump exercise declined to baseline values after only 6 months of detraining in premenopausal women (Fig. 3). A parallel response was also observed in leg strength and power (Fig. 4). In our 2-yr observations of gymnasts (98), bone at the hip, spine, and whole body consistently increased over the training seasons and decreased in the off-season (Fig. 5). Only a few studies have addressed the skeletal response to loading in the years just prior to menopause. Results have indicated that perimenopausal women who exercise will maintain BMD at loaded sites to a greater extent than those who do not (99,100).

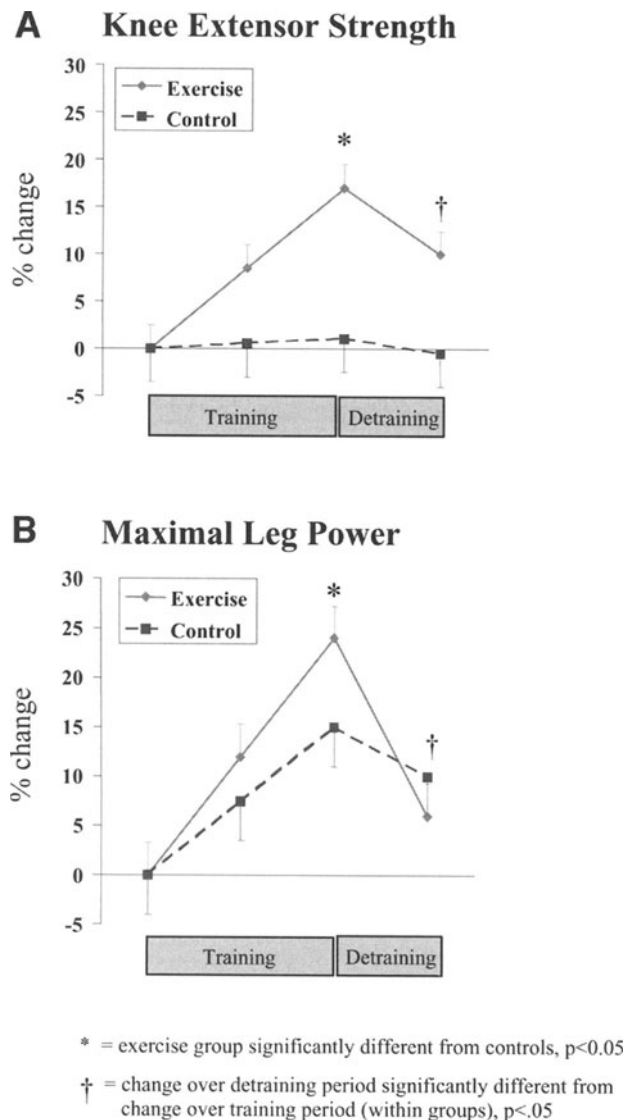


Fig. 4. Percent changes in lower extremity strength and power in mature premenopausal women across 12 mo of training and 6 mo of detraining. (mean \pm SEM) for A) knee extensor strength and B) leg power. Reprinted with permission, Winters & Snow, JBMR, 2000.

POSTMENOPAUSAL WOMEN

The low estrogen levels that accompany menopause represent a powerful confounding factor when studying exercise effects on bone. Estrogen withdrawal induces rapid bone loss in the years immediately following menopause and exercise interventions that combine both early and late postmenopausal women (the norm in these studies) may fail to distinguish the factor imparting the greatest effect on bone. The three reported investigations specifically targeting *early* postmenopausal women who were estrogen-deplete concluded: 1) resistance exercise benefited the lumbar spine but provided insufficient stimulus to prevent hormone-related bone loss at other skeletal sites (101), and 2) both high

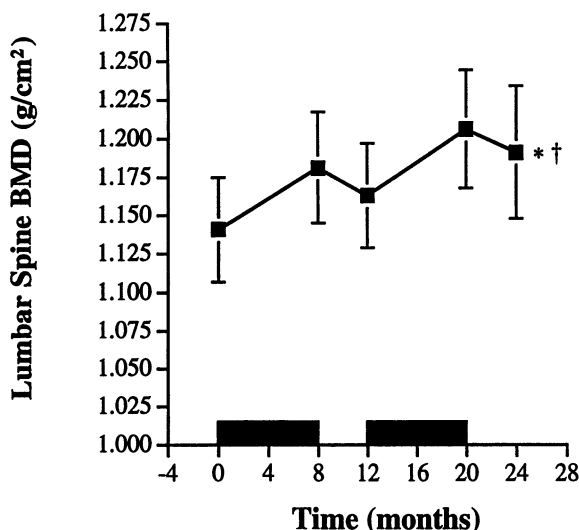


Fig. 5. Changes in spine bone mineral density over 24 mo in intercollegiate gymnasts. There was a significant seasonal quartic trend for increases and decreases in spine bone mineral density (†) as well as a significant linear trend (*). Spine BMD increased an average of 3.6% during the 8-mo training seasons and decreased an average of 1.5% in the off-season. The overall increase in 24 mo was 4.3%. Black bars indicate the timing of the competitive training seasons. Data are expressed as mean \pm SEM. Reprinted with permission, Snow et al., *Calcified Tissue International*, 2001.

and low impact exercise maintained spine BMD (102), and 3) high versus moderate intensity resistance training increased trochanteric BMD but had no effect on spine BMD (31).

In older postmenopausal women, resistance training programs of 9–24 mo duration in estrogen-deplete postmenopausal women are generally associated with an increase or maintenance of BMD compared to losses in control subjects at the whole body (26), lumbar spine (26,101,103–105), proximal femur (25,103,105), and radius (25), although not without exception (27,106,107). Weight bearing aerobic exercise interventions of 7–18 mo duration are also generally associated with increases or maintenance of BMD compared to losses in control subjects at the whole body (26,108), lumbar spine (11,102,108–110), proximal femur (26,108), radius (110), and calcaneus (111,112).

Due to the lower load magnitude associated with activity of lower intensity, these exercise programs do not promote bone gain or reduce loss in postmenopausal women. It is generally agreed that walking alone is not an effective strategy for osteoporosis prevention in postmenopausal women (113). An exception to this is the report of Hatori and colleagues (109) who found that 7 mo of walking 3 times/wk at speeds above the anaerobic threshold increased lumbar spine BMD in postmenopausal women. The increased forces associated with walking at higher speeds combined with lower initial BMD values may explain the positive response. In a study of walking resistance exercise, osteoporotic women improved vertebral trabecular BMC compared to controls who lost bone (114). In this case, the higher loads at the spine from resistance exercise were osteogenic. By contrast, 12 mo of unloaded exercise in waist-deep water did not prevent spine bone loss or improve femoral BMD in osteoporotic women, despite changes in other functional fitness parameters (115).

The length of participation in weight bearing exercise may be of central importance in a bone response in older adults. For example, although no change in femoral neck

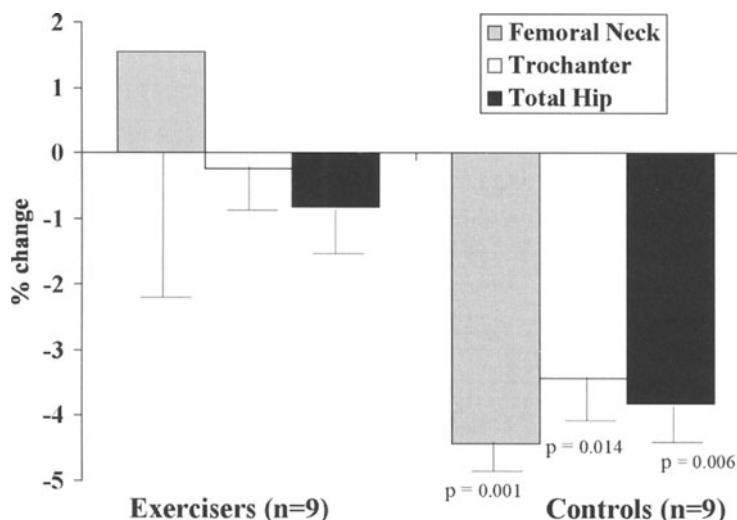


Fig. 6. Percent changes in BMD at the femoral neck, trochanter and total hip in postmenopausal women exercisers and controls after 5 yr. Changes for exercisers were $1.54\% \pm 2.37$ at the femoral neck, $-0.24 \pm 1.02\%$ at the trochanter and $-0.82 \pm 1.04\%$ at the total hip whereas controls decreased $4.43 \pm 0.93\%$ at the femoral neck, $3.43 \pm 1.09\%$ at the trochanter and 3.80 ± 1.03 at the total hip. Decreases in controls are significantly different from zero (unpaired *t*-tests). Data are presented as means \pm SEM. Reprinted with permission, Snow et al., *J Gerontol* 2000;55A:M1–3.

BMD in post menopausal women was observed from nine months of jump plus resistance exercise wearing weighted vests (20), after 5 yr of participation in this program using weighted vests, significant bone loss of more than 4% at the hip was prevented (13) (Fig. 6). This response is similar to or better than that of estrogen and far more acceptable to many postmenopausal women who are reluctant to take estrogen. In fact, over 60% of postmenopausal women who begin estrogen discontinue therapy within 1 yr (116).

Given the importance of site-specificity, it is not surprising that weight bearing exercise does not increase forearm BMD in postmenopausal women (26,117). However, specific loading of high rate and magnitude, resulted in higher forearm bone density in osteoporotic, postmenopausal women after only five months (118,119).

HORMONE REPLACEMENT AND EXERCISE

Alternative approaches to hormone replacement therapy (HRT) for postmenopausal women, has prompted research that examines the efficacy of exercise in comparison to, and in combination with HRT. In some reports, exercise enhances the bone maintenance effect of HRT. For example, in a study of one year of resistance exercise, surgically-menopausal women who were estrogen-replaced increased spine, total body and radial mid-shaft BMD in compared to estrogen-replaced, non-exercising controls who maintained BMD (120). Similarly, the interaction of HRT and nine months of weight bearing exercise (walking, jogging, stairs) resulted in greater increases in total body and lumbar spine BMD in 60–72-yr-old postmenopausal women than exercise or HRT alone (121).

By contrast, other studies report no interaction between exercise and HRT. For example, a 3-h/wk program of resistance exercise plus walking or running for 1 yr did not enhance the positive effect of estrogen supplementation on lumbar vertebral or femoral neck BMD

in postmenopausal women (*122*). Similar results were observed at the lumbar spine and hip in early postmenopausal women on HRT, despite a positive effect of 3-h/wk exercise on BMD in a placebo group (*123*). In these studies, a longer intervention period and higher load magnitudes are likely necessary for more positive outcomes.

EXERCISE INTERVENTIONS IN YOUNG AND OLDER ADULT MEN

Although there are few longitudinal studies in men, the response of the male skeleton to exercise is similar to that of women and is not complicated by an abrupt disruption in reproductive hormones in adulthood. After 14 wk of intensive physical training male army recruits increased leg BMC by 12% (*76,124*) and those with the lowest initial values gained the greatest amount. Those who temporarily stopped training due to stress fracture also gained bone density, but to a lesser degree (5%). However, 10% of recruits lost bone density, either due to incomplete remodeling owing to the short observation period or to abnormal remodeling due to fatigue and inadequate rest intervals from training intensity and volume. Furthermore, the influence of training intensity on bone response becomes apparent when results of the army trials are compared with those of recreational athletes (*85*). In contrast to army recruits, men aged 25–52 failed to gain bone at the spine, humerus, femur, calcaneus or forearm following 3 mo, of either walking (3 km, 5 d/wk), or running (5 km, 3 d/wk). Thus, bone mineral gains observed in the army recruits reflect both the higher load magnitudes from basic training and the younger age of the subjects. The only other report in young men, nine months of marathon training, resulted in significantly higher calcaneal BMC in the runners than nonrunners (*111*). Furthermore, there was a positive association between average distance run and percent change in BMC.

There are few exercise interventions in older men. In a study of six men aged 50–73 yr who performed step and jumping exercise for months, Welsh and Rutherford (*108*) reported a significant increase in trochanteric BMD. In a recent study, Maddalozzo and Snow (*31*) examined the effects of 6 mo of two different resistance training programs on bone mass in older men (*31*). Following a 3-mo observational control period, 20 healthy older men (mean age = 54.6 yr) were randomly assigned to either a high-intensity standing free-weight program or to a moderate-intensity seated resistance-training program. High intensity training resulted in a 2% gain in lumbar spine BMD in men whereas moderate intensity training produced no changes at this site. However, increases in bone mass were observed at the greater trochanter for men regardless of training intensity. Both training programs resulted in improvements in total body strength (38%) and lean mass (4.1%). Continued research efforts should be directed at this population who are increasingly at risk for osteoporosis as it appears that improvements in both muscle and bone are observed in older men following specific exercise.

CALCIUM AND EXERCISE

The permissive action of calcium in enhancing the effect of exercise on BMD is somewhat controversial. In a review of 17 trials, Specker (*125*) concluded that an intake of 1000 mg/d of calcium is necessary in order to observe a skeletal response to exercise and there is some evidence that the combination of calcium supplementation and exercise is more effective for a bone response in postmenopausal women than calcium supplementation alone (*11,126,127*).

In contrast to the positive reports, a recent cross-sectional study of 422 women found that even though high levels of physical activity calcium intake were associated with a higher total body BMC than low activity levels and low calcium intake, there was no interaction between exercise and calcium (65). In an exercise plus calcium intervention study, 2 yr of combined aerobics and weight training increased the BMD in young women, but calcium supplementation neither enhanced the exercise benefit nor improved BMD in the absence of exercise (95). Thus, although exercise likely provides a greater stimulus to bone than does calcium, adequate calcium intake is recommended to provide the building blocks for exercise-induce gains in BMD.

Exercise-Related Geometric Adaptation

Exercise has the potential to improve bone strength by altering geometric properties through increasing cross-sectional area and cross-sectional moment of inertia. Femoral mid-shaft cortical thickness increased in prepubertal boys after eight months of weight bearing activity (54). In postmenopausal women, site-specific exercise resulted in structural changes that were independent of BMC increase, such as increased cortical bone area (128). Athletes who preferentially load their dominant limb exhibit both expanded diaphyseal diameters and increased BMD compared to nonathletes. Krahl and colleagues (129) observed differences in diameter and length of playing arm ulnae of tennis players compared to the contralateral arms. The second metacarpals of playing hands were also wider and longer than in contralateral hands, whereas no differences were observed between limbs of controls. Similarly, Dalen and associates (50) observed a 27% difference in cortical cross sectional area between left and right humeri of tennis players compared to a nonsignificant 5% difference in controls. Differences between playing and non-playing arm humeral cortical wall thickness, length, width and cross sectional moment of inertia have likewise been observed by others (51). It is important to note that these geometric changes have been reported for cortical, but not trabecular bone. Since the clinically relevant fracture sites are the hip and spine, more work should be directed toward examining geometric changes at these important bone sites. In a randomized controlled jumping intervention, Fuchs et al. (14) reported increases in femoral neck bone area in prepubertal children. This increased femoral neck projected area indicates greater femoral neck cross-sectional area as evidenced by a strong association ($r^2 = 0.85$) between the projected DXA-derived measure and estimated cross-sectional area-derived measurements from DXA (Bone Research Laboratory, unpublished observations).

CONCLUSIONS

Physical activity through specific exercise regimens increases peak bone mass and slows or prevents age-related bone loss. The most osteogenic activities are those that involve high magnitude and loading rates. Such loads are likely to be most effective when accompanied by adequate calcium consumption and, for hypoestrogenic women, hormone supplementation. Individuals with low baseline values of bone density or activity may experience the greatest gains in bone. Exercise-induced changes in bone geometry are still poorly understood, but efforts should be directed at determining the effect of increasing bone geometry, particularly at high-risk trabecular sites such as the hip and spine. In order to develop an exercise prescription

for increasing bone mass, it will be necessary to fully develop a dose response surface by applying quantified loads to the hip and spine and determining not only the changes in bone density, but also estimates (using theoretical models) of the structural consequences of these changes.

HORMONE RESPONSE TO INTENSE EXERCISE

Women

Exercise-associated amenorrhea occurs in some young and mature premenopausal women from repeated, intense, exercise training. While low body fat is assumed to be primary in the etiology of exercise-associated amenorrhea, it has become clear that a disruption in the hypothalamic-pituitary-thyroid (H-P-T) axis from reduced energy availability is also a contributing factor (130). Based on observations in female athletes, Loucks and coworkers report that energy deficiency suppresses reproductive function secondarily to thyroid function and thus exercise-associated amenorrhea may be prevented or reversed by increasing energy availability through dietary changes, without alterations in training (131).

Another potentially important factor in the etiology of exercise associated amenorrhea is that of the peptide hormone, leptin. There is evidence that the interaction between nutrition and reproduction may be driven, in part, by leptin (132,133). Since amenorrheic athletes report lower dietary intakes than would be required by their level of activity and leptin levels are lower in these individuals (134,135), it is plausible that the hormone may be central in the underlying mechanism of the syndrome. In a recent report of amenorrheic and eumenorrheic in athletes, Farah and colleagues (136) found that leptin was associated with triiodothyronine and insulin but not reproductive hormones or energy availability. They concluded that leptin is not directly regulated by estrogen but that it may mediate the interdependence between reproductive function and energy availability.

Since estrogen levels are reduced, the consequence of exercise-associated amenorrhea is bone loss (84,137). The “female athlete triad” describes the combined conditions of excessive dietary restraint, hormonal disturbance and bone loss in female athletes. In all but cases of extreme loading, the positive effect of exercise on bone cannot to offset the negative effects of inadequate energy intake high intensity, high volume exercise training. The exception to this are gymnasts, who load their bones at very high loading rates that, despite a high prevalence of menstrual disturbance, have bone density values well above normal (29). Long distance runners, on the other hand, who load their skeletons at much lower loading rates, are not protected from amenorrhea-related bone loss. Although there are individual differences, the loss of bone mass in amenorrheic distance runners increase their risk of stress fracture and premature osteoporosis than their eumenorrheic running counterparts (138).

Oral contraceptives may offset bone loss in athletes with menstrual dysfunction. However, there are insufficient data to fully corroborate this effect (139). Keen and Drinkwater (140) reported that initiating oral contraceptive use approx 8 yr after athletic oligo- or amenorrhea did not improve bone mass, concluding that intervention should begin at the onset of dysfunction in order to prevent significant loss.

Men

Intense training is not associated with severe alterations in reproductive hormones in men. Male athletes exercising at a range of intensities have serum concentrations of testosterone that lie within the normal range (77,78,89,141,142) including adolescents (143).

However, a degree of subtle hormonal perturbation are apparent in some athletes. Smith and Rutherford (89) reported that, although in the normal range, serum total testosterone was significantly lower in triathletes than controls, but not rowers. Further, total serum testosterone, non-sex hormone binding globulin (SHBG)-bound testosterone, and free testosterone concentrations in men running more than 64 km/wk were found to be 83, 69.5, and 68.1% that of controls, respectively (144). Prolactin concentrations were also significantly lower in runners than controls. Others have similarly observed that resting and free testosterone concentrations of trained athletes are 68.8 and 72.6% that of controls (145). Age may influence the effect as, elderly endurance athletes have significantly greater levels of SHBG than controls whereas younger athletes demonstrate no differences compared to controls (78,146).

Whether hormones potentiate the effect of exercise on bone in men is relatively unexamined. Suominen and Rakkila (78) reported a negative correlation between BMD and SHBG in older endurance athletes but no relationship of BMD with testosterone. Further, the addition of self-administered anabolic steroids (testosterone: 193.75 ± 147.82 mg/wk) to high intensity body building training does not stimulate greater osteoblastic activity or bone formation than exercise alone (147). Four months of progressive resistance exercise training 4 d/wk, with or without growth hormone supplementation, did not significantly increase whole body, spine or proximal femur BMD in elderly men (mean age 67) with normal BMD (148). Similarly, the addition of recombinant human growth hormone to 6 mo of resistance exercise training resulted in no change in BMD of older men (149,150). Additional work is necessary to elucidate the relationship between exercise, hormone status and bone metabolism in men.

EXERCISE, FALLING, AND FRACTURE

Most research into the role of physical activity in decreasing fall risk and fracture has been conducted within an epidemiological context, rather than through exercise intervention. Since falls and fractures are relatively infrequent events, prospective trials are difficult in light of the large sample size and long follow-up period necessary to observe a significant effect for physical activity.

Fractures

In general, available studies support a protective effect of physical activity on the risk of fracture, especially those at the hip (151–154). Specifically, the Study of Osteoporotic Fractures, a large, prospective, community-based, observational study of healthy, older, Caucasian women, found that moderate to vigorous activity was associated with reductions in hip and vertebral fracture incidence (151). Following exercise interventions, individuals with normal mobility improve bone mass, VO_{2max} , well-being, stamina, mobility, and pain tolerance, with no higher incidence of fracture (155).

Given that over 90% of hip fractures occur from a fall (156,157), it is important to characterize the relationship between physical activity and falls, especially injurious falls that are most likely to precipitate hip fracture (156,158,159). Lateral instability, muscle weakness of the lower extremities, and inability to perform a tandem gait independently predict hip fracture and falls (160–163). These intrinsic risk factors for falls respond favorably to exercise intervention (20,164,165). However, while falls to the side raise the risk of hip fracture more than sixfold, little is known about the predictors

of side falls and, as far as we are aware, exercise interventions have not been used to reduce side fall risk. While over 50% of vertebral fractures are associated with backward falls (166), associations between falls, physical activity and vertebral fracture are not well-defined (151).

Falls

As mentioned previously, exercise is unique in that it can affect both the numerator and denominator of the factor of risk. Discussion thus far has focused on exercise as a means of altering the denominator of the factor of risk, that is, on increasing fracture load by influencing bone density. However, exercise is also important for fall prevention and can reduce the numerator either by preventing a fall entirely or by lowering the applied load of those falls that do occur through improved neuromuscular responses. The most important mechanisms involved in preventing severe trauma during a fall are likely the eccentric contraction of lower extremity muscles during descent and the successful use of the arms to help break the fall.

Muscle weakness, postural stability and functional mobility are important risk factors for falls and hip fracture (160). Since exercise promotes and maintains muscle strength, balance and mobility, it is thus a highly appropriate strategy for reducing osteoporosis-related fractures (167,168). High intensity resistance training 1, 2, and 3 times per week for 24 wk significantly increased total body muscle strength and neuromuscular performance in community-dwelling men and women aged 65–79 yr (169). The lack of difference in improvement found between subjects regardless of number of training sessions per week suggests that enhanced strength and neuromuscular function in older adults is attainable with less commitment of time than previously thought.

Most exercise interventions have focused on decreasing fall risk rather than reducing fracture incidence. These interventions have produced improvements in performance variables related to fall risk. Specifically, dramatic increases in muscle strength, functional mobility such as stair climbing and rising from a chair, gait speed and even confidence in movement skills have been documented (165,170,171). However, not all muscle-building interventions demonstrated balance improvements along with strength gains (170). Inconsistencies in the data are probably due to variation in the modes of exercise. For example, a range of exercise intensities (low to high) and seated versus standing modes have been reported and these variations likely determine whether or not the exercise outcomes transfer to activities of daily living. In community-dwelling women, muscle strength and power gains over 9 mo of training were predictive of improvements in lateral stability while the more elderly group experienced improvements in gait parameters. Because of the association of falling to the side and the risk of hip fracture, improvements in lateral stability may reduce fracture risk.

Exercise interventions with falls and injurious falls as primary outcomes are limited and the results inconsistent. Lord and coworkers (172) reported improvements in strength and balance in elderly women but no change in incidence of falls after 12 mo of exercise that included resistance. By contrast, Campbell (164) found that a multifactorial exercise intervention involving muscle building plus walking exercise reduced injurious and noninjurious falls by 40% in elderly women. However, this study required home visits by physical therapists and it is not known which component(s) of the program (muscle building, walking or the two combined) were most potent for reducing falls. Lastly, data from the

FICSIT trials (Frailty and Injuries: Cooperative Studies of Intervention Techniques) indicate that activities that are most beneficial for reducing incidence of falls include those that result in muscle strength gains and dynamic balance improvements (173).

CONCLUSION

The strong positive effect of exercise on muscle strength balance and mobility indicates that it is an important strategy for preventing osteoporosis related fractures by improving or maintaining neuromuscular function, thus, reducing the numerator of the factor of risk in older adults.

CONCLUSIONS AND RECOMMENDATIONS

While much has been achieved in our understanding and use of exercise for the prevention of age-related fractures, many questions and challenges remain. For instance we have only a limited understanding of the molecular, cellular and tissue-level mechanisms by which bone remodeling occurs in response to exercise. We also know little about the relative importance of mechanical, electrochemical, endocrine and genetic factors and how these interact to potentiate or blunt the exercise response in bone over the life span. Furthermore, there is uncertainty and even controversy as to whether bone cells respond directly to mechanical strain, to electrochemical effects from fluid flow, or to hormonal influences or some combination of these. With respect to strain, we also know little about whether bone responds more favorably to compression, tension or shear, or to their combination. Some have suggested that bone remodeling in response to exercise results from the accumulation of local microcracks and the accretion of bone from their repair. How these mechanisms relate to the temporal sequence of the bone remodeling cycle is also unclear.

In the face of these uncertainties about bone remodeling mechanisms and their changes with age, it is not surprising that exercise interventions focused on bone reflect such a wide range of exercise modes, loading magnitudes, rates, training session frequencies and within session number of repetitions. Sorting out the relative importance of these variables and providing a theoretical framework for understanding and extending the reach of experimental results in exercise studies also remains a major challenge. While Whalen and colleagues (174) have made a major contribution with their power law model incorporating load magnitude and number of repetitions, it is becoming increasingly clear that, rate effects must also be included in these empirical models. The eventual goal, of course, is to generate dose-response relationships specific to particular exercise modes and appropriate for the age group of interest.

Exercise interventions of relatively short term (6–18 mo) have demonstrated that activities of high magnitude and high loading rate promote bone gain in children and adults. These exercises include impact activities (e.g., jumping) and resistance programs such as weight training and the use of weighted vests. There is also evidence that long-term participation in weighted vest plus low impact jumping significantly reduces risk factors for falls (20) and prevents bone loss of more than 4% at the hip in older postmenopausal women (13). Although walking at moderate intensity has not been shown to increase bone mass in these relatively short study interventions, a *lifetime* of walking is

likely to be beneficial to the skeleton. In a recent report, individuals who engaged in lifetime weight-bearing activities such as walking had higher bone mass compared to those who were less active (175).

Even as we better understand the relative benefits of interventions aimed at: 1) increasing bone mass in the young, 2) reducing age-related bone loss in midlife, and 3) improving neuromuscular function such as balance, strength, power and response time, it is likely that there will be continued efforts to use exercise as a means for minimizing bone loss and maintaining neuromuscular capabilities in the elderly at highest risk of fracture. In this population, particular care must be taken to maintain a balance between safety and efficacy, since the exercise intervention itself (through increasing activity levels) presents not only the possibility of skeletal and neuromuscular benefit, but also an increased risk of fracture. As we achieve a better understanding of the mechanisms involved in the exercise-induced remodeling of bone and how it changes in the aging skeleton, there will inevitably be attempts to find those combinations of exercise mode, load magnitude, loading rate and numbers of repetitions that will prove most effective in the elderly. Such attempts to move bone beyond the “lazy zone” in the aging skeleton should only be pursued with as clear an understanding as possible of the forces applied to the skeleton region at risk and their relationship to the tolerance limits of these regions. Fortunately, through a combination of dynamic force analyses at the hip and spine and *ex vivo* measurements in cadaveric bone, it is possible to determine the factor of risk for most of the exercise activities that might be used in exercise studies in the elderly. Understanding and maintaining a safe value of the factor of risk for a specific exercise program is crucial. Until such understanding is achieved, caution is warranted in the use of overload concepts, particularly in the frail elderly.

While high magnitude *impact* activities are recommended for increasing bone mass of the younger, more robust skeleton, they are not recommended for elderly with advanced osteoporosis. Such individuals, with or without a history of vertebral compression fractures, should not engage in jumping activities or deep forward trunk flexion exercises such as rowing, toe touching and full sit ups. Before initiating a program of high intensity, elderly individuals should consider a bone density evaluation. Resistance training programs that promote balance and upper and lower body muscle strength, will be of greatest benefit to osteoporotic individuals by reducing the risk of falling. Any individual undertaking a new program should begin slowly with careful attention to exercise form and appropriate progressions. Exercises that produce severe joint pain or muscle soreness of more than 3 d should be discontinued until exercise of lower intensity can be tolerated.

Thus, physical activity has the potential to reduce the development of osteoporosis and fragility fractures by: 1) increasing peak bone mass, 2) maintaining or increasing adult bone mass, and 3) reducing the risk and incidence of falls. Because these three factors are age-specific, exercise prescription for osteoporosis-related fractures differ across the lifespan. During youth, emphasis should be on increasing bone by introducing high impact activities. In young and middle adulthood, activities should be directed on maintaining and/or increasing bone mass through weight bearing exercise of sufficient magnitude to force bone out of the lazy zone (activities that impart forces of >2.5 body weights at the hip and the spine). For old age, exercise programs should emphasize activities that challenge the neuromuscular system and use resistance for building muscle and bone.

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The Physiology and Cell Biology of Calcium Transport in Relation to the Development of Osteoporosis

Richard L. Prince, MD and Paul Glendenning, PhD

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INTRODUCTION

The vital role that calcium plays as a second messenger in cell signaling processes highlights its importance in a wide range of cellular activities and its fundamental importance to the sustenance of health. In addition, calcium has a specific role in the conduction of action potential along nerves and in the coupling of excitation and contraction in striated and cardiac muscle. These concepts are mentioned in order to acknowledge the importance of calcium in many physiological processes, but the principle focus of this chapter will be on the mechanisms regulating extracellular calcium levels and the ways in which they may affect bone function. In the short term, extracellular calcium balance is far more important to the survival of the individual than total body calcium stores. Since the skeleton contains most of the total body calcium (1–2 kg), it is this compartment that compensates for any reduction in extracellular calcium and it does so at the expense of bone mineral accretion. Consequently, it is the tension between the requirements of separate body compartments that sets the scene for the importance of calcium nutrition in the prevention and treatment of age related osteoporosis.

At the tissue level, the principle organs involved in extracellular calcium homeostasis are bone, gut and kidney. These structures regulate the flow of calcium into or out of the extracellular space (Fig. 1). It is critically important that calcium is continually cycling in and out of the blood perfusing these organs. In the kidney 98% of the calcium filtered at the glomerulus is reabsorbed, approximating 150 mmol/d. In the bone, 5 to 10 mmol/d of

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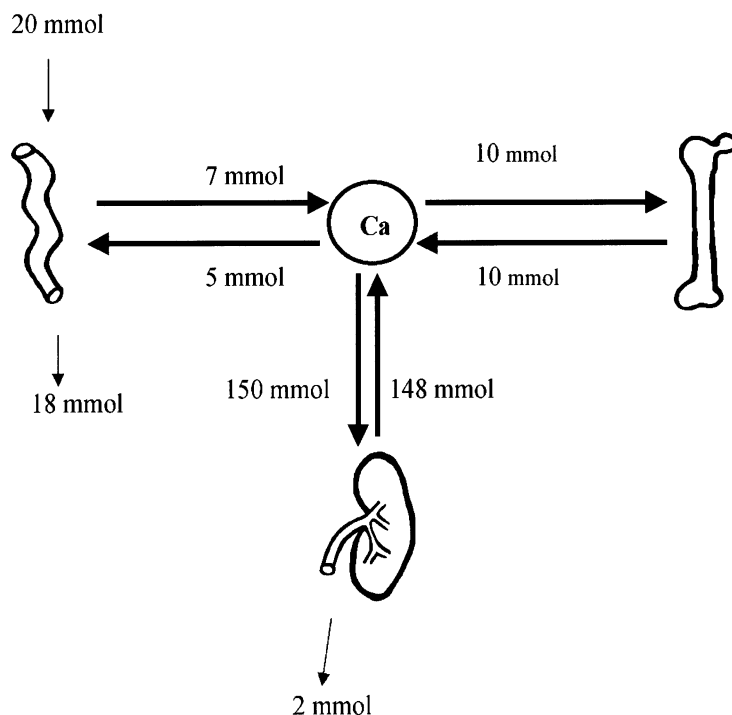


Fig. 1. Organs involved in calcium transport: patient in calcium balance.

calcium cycles into and out of the skeleton. In the bowel, about 4 mmol/d of calcium is secreted into the lumen from the exocrine pancreas, bile and intestinal enterocytes. Food contributes about 20 mmol of calcium to intestinal calcium, and approx 7.0 mmol of calcium is reabsorbed in the gut per day. Thus, calcium is in a state of continuous flux into and out of the principle organs involved in extracellular calcium homeostasis. Similarly, calcium is continually moving in and out of all the cells of the body. Thus the critical issue in the control of this system is to regulate the relative activity of the various organs and cells in order to maintain a constant internal cellular environment.

A detailed understanding of cell biology is required to appreciate the principal mechanisms involved in transmembrane calcium transportation. A unifying concept is the similarity between calcium transport mechanisms in the kidney distal tubule, small intestinal epithelium and bone. These calcium transport mechanisms exist to adapt to times of nutritional calcium deprivation as well as to times of calcium sufficiency.

Hormonal Regulators of Calcium Homeostasis

Two hormones play a major role in regulating extracellular calcium concentrations (Fig. 2). The concentrations of both parathyroid hormone (PTH) and calcitriol (1,25 dihydroxyvitamin D₃), are regulated by the concentration of calcium in the extracellular fluid, probably via a G-protein linked calcium receptor in the membrane of parathyroid hormone cells and proximal tubule cells of the kidney respectively (1). There is a feedback interaction between PTH and calcitriol; PTH stimulates calcitriol synthesis and calcitriol reduces PTH levels. (2). They also work in a coordinated way to influence the movement of calcium across membranes in the bowel, kidney or bone to maintain the calcium concentrations in the extracellular compartment. For example, if dietary cal-

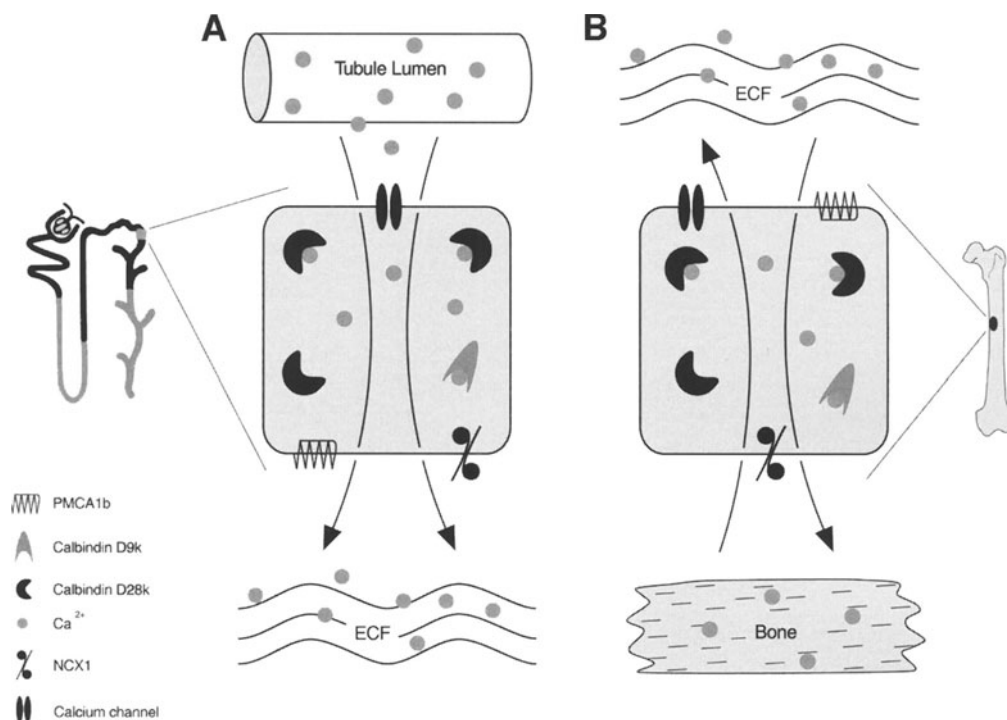


Fig. 2. Comparison of calcium transport in the kidney distal tubule and osteoblast. Calcium transport is unidirectional in the kidney distal tubule but bi-directional in the osteoblast. PMCA1b is expressed on the apical plasma membrane of osteoblasts, but on the basolateral membrane in the kidney distal tubule. The remaining features of transmembrane calcium transport in both cells are distinctly similar.

cium intake declines the coordinated action of PTH and calcitriol increases calcium influx from the bowel, urine and bone compartments to ensure stability of extracellular levels (3). Estrogen deficiency plays a central role in the development of postmenopausal osteoporosis and so there is a *prima facie* case for the involvement of estrogen in the regulation of calcium transport across membranes either directly or indirectly. There is evidence that estrogen is important in determining the rate of flux of calcium into and out of the bone, kidney tubule and bowel lumen and thus can indirectly determine circulating concentrations of PTH and calcitriol (4).

Proteins Involved in Calcium Transport

To understand the individual proteins involved in active transmembrane calcium transport, the kidney distal tubule epithelial cell can be considered as an example. The salient features of that system are similar to those in the other two tissues involved in calcium transport (intestine and bone). The transcellular, diffusional-active transport of calcium reabsorption in the kidney distal tubule involves three separate subcellular components (Fig. 3). First, calcium enters the cell across the apical plasma membrane through discrete calcium channels (6) including the newly described epithelial calcium channel (ECaC) (7). ECaC is a 83 kD protein localized to the apical membrane of enterocytes and distal tubule

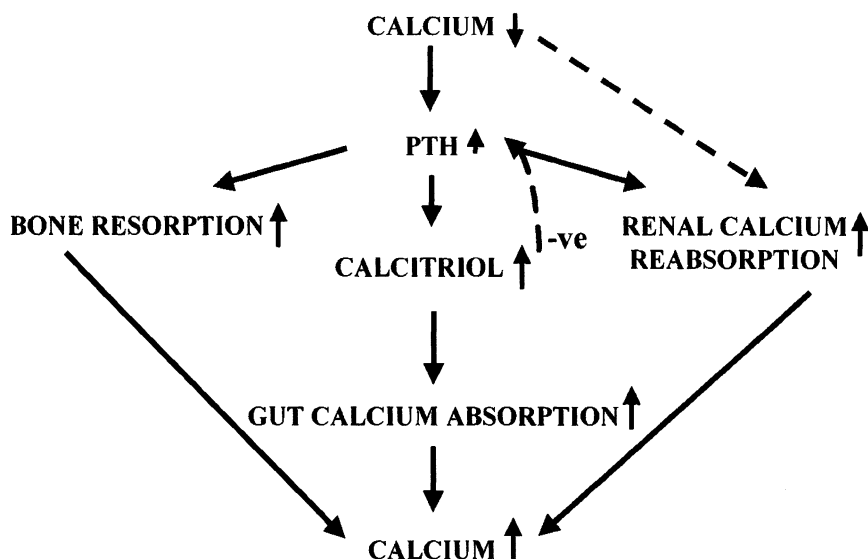


Fig. 3. Feedback regulation of calcium homeostasis: effects of PTH and calcitriol.

cells (7). The protein contains six transmembrane domains with the putative calcium transport region occurring between transmembrane domains five and six.

Second, the presence of two intracellular calcium binding proteins, appropriately termed calbindins, act to expedite transcellular calcium movement and prompt ion delivery to the opposing, basolateral membrane (8). Calbindin-D_{28K} is a 28 kD cytoplasmic protein of approximately 261 amino acid residues. It is a member of the EF-hand loop helix proteins that bind calcium with high affinity. Each molecule has six high affinity calcium binding sites although only four are active (9). The distribution of Calbindin-D_{28K} has been shown to be quite widespread in mammalian tissue. In addition to tissue typically involved in calcium transport (the intestinal enterocyte, the distal convoluted tubule and collecting duct of the kidney, the osteoblast), calbindin-D_{28K} expression has also been found in neurones, pancreatic islet cells and in testes.

Calbindin-D_{9K} is another member of the high affinity calcium binding proteins containing two EF-hand structures. It has little sequence homology with Calbindin-D_{28K}, and consists of about 79 amino acid residues and two high affinity calcium binding sites (9). The tissue distribution of Calbindin-D_{9K} is similar to that of Calbindin-D_{28K}.

Last, calcium efflux at the basolateral membrane involves an active transport process. Two mechanisms exist on the basolateral membrane to subserve this purpose: the plasma membrane calcium ATPase (PMCA) and the Na⁺/Ca²⁺ (NCX) exchanger (10,11). PMCA is a member of the P-type ATPase family, is calmodulin-dependent and forms a phosphorylated intermediate (12). Calmodulin affinity chromatography was first utilized to separate and purify PMCA (13). At least four different genes code for PMCA proteins (PMCA1-4) and posttranscriptional modification of each primary gene transcript produces distinct and uniquely different isoforms which create a diversity of functional consequences (14). The 130–140 kD polypeptides share about 75% of amino acids in several highly conserved regions (14). The site of ATP binding and the site of phosphorylation represent two of these highly conserved regions. NCX is a secondary basolateral membrane transport system. It uses the electrochemical gradient produced by sodium ATPase activity (11) to move calcium across the membrane. It is a 970 amino acid protein with a primary structure that

contains 11 transmembrane spanning regions and a large cytoplasmic loop between transmembrane segments 6 and 7. NCX is particularly abundant in cells that handle large fluxes of calcium across their membranes such as contractile and neuronal cells. The orientation of NCX is determined by the interaction of two inwardly directed electrochemical gradients generated by plasma membrane sodium and calcium pumps. This electrochemical gradient is determined by the net activity of PMCA, the sodium ATPase pump, sequestration of calcium within intracellular organelles and the membrane potential difference. Recent evidence directly implicates estrogen and testosterone in stimulation of the plasma membrane calcium ATPase PMCA, a critical regulator of transmembrane calcium flux (5).

THE ROLE OF THE KIDNEY IN EXTRACELLULAR CALCIUM BALANCE

Physiology of Calcium Handling in the Nephron

The kidneys filter approx 100–200 mmol of calcium per 24 h of which about 98% is reabsorbed. Because of the high rate at which calcium is cycling across the renal tubular membrane it is possible for subtle variations in the rate of reabsorption to have profound effects on extracellular calcium balance. Approximately 70% of calcium reabsorption occurs in the proximal tubule (15) and is largely passive, voltage-dependent and associated with active reabsorption of sodium, glucose and other solutes. In the kidney, paracellular calcium transport is regulated by the extracellular ionized calcium concentration that acts on the calcium sensing receptor (CaSR). The CaSR has recently been cloned from human (16) and rat (17) kidney.

In the distal kidney tubule, sodium and calcium reabsorption can be uncoupled, for instance with thiazide diuretics (17a). These in vivo observations are now being examined in vitro where a role for each calcium transport protein in kidney distal tubule calcium transportation is being developed.

At least three dietary constituents influence renal calcium excretion: Sodium, protein and acid. Sodium competes with calcium for reabsorption in the proximal and distal tubule as evidenced by the strong association between sodium and calcium excretion (18). These data seem specific for sodium chloride since other sodium salts, such as bicarbonate or citrate, do not alter renal calcium excretion (19,20). In a 2-yr, prospective, epidemiological study of the effects of sodium intake on bone mass in elderly postmenopausal women, higher sodium intake was associated with a greater degree of bone loss (21). In the same cohort, high calcium intake prevented sodium induced bone loss and an analytical model that included both minerals predicted change in bone mass better than a consideration of either alone.

Dietary protein intake increases renal calcium excretion (22). Although increased protein intake may increase glomerular filtration rate, the hypercalciuric effect appears to be related to excretion of fixed organic acid as a result of the metabolism of, in particular, sulfur containing amino acids. Certainly the effect can be reversed by increasing dietary alkali intake (23). In population studies there was no excess risk of renal calculia at protein intakes over 76 g/d compared to intakes under 42 g/d (24). In old age there is evidence that a protein supplement will actually improve bone density and clinical outcomes after hip fracture (25). Certainly there is a positive association between protein intake and IGF1 levels in postmenopausal women (26).

The effect of alkali to reduce renal calcium excretion is well described and has been attributed to effects on bone resorption and renal calcium excretion (27,28). The mecha-

nism is uncertain and indeed it is likely that effects on both bone and kidney may be linked as a method of buffering excess food acid.

Cellular Localization of Renal Epithelial Calcium Transport Proteins

Both PMCA and NCX are expressed on the basolateral membrane of the kidney distal tubule (29–31). The availability of isogene-specific antibodies to PMCA revealed the presence of PMCA1 and PMCA4 but (not PMCA2 and PMCA3) isogenes in human kidney crude plasma membranes (32). This finding was verified using crude microsomal preparations from human kidney and monoclonal antibodies to PMCA1, PMCA4a and PMCA4b (33). EcaC is an apical influx channel in the distal kidney tubule (7). The calbindins exist within the cytosol of the epithelial cells lining the distal kidney tubule (31). Recent evidence for the stimulation of the Na^+ - H^+ antiporter in the distal kidney tubule by calbindin $\text{D}_{28\text{K}}$ (thereby reducing intracellular sodium and consequently increasing calcium transport) highlights one mechanism by which calbindin $\text{D}_{28\text{K}}$ could influence NCX1 activity in this cell (34). Chronic metabolic acidosis has also been noted to increase calbindin $\text{D}_{28\text{K}}$ expression in rat kidney distal tubule (35). Thus, all four transport proteins probably act in a coordinated fashion to regulate calcium excretion in the distal kidney tubule.

Hormonal Regulation of Renal Calcium Transport

PMCA1b, calbindin $\text{D}_{28\text{K}}$ and calbindin $\text{D}_{9\text{K}}$ are upregulated by calcitriol. Vitamin D deficiency has no effect on NCX1 activity (36). Although several isoforms of PMCA exist within the kidney, only one (PMCA1b) appears to be hormonally responsive. PMCA1b is regulated by calcitriol in rabbit and bovine distal kidney tubule cells (37,38) and it is probably this effect that accounts for the increased calcium reabsorption demonstrable in calcitriol-treated vitamin D-deficient rabbits (36). Calbindin $\text{D}_{28\text{K}}$ and calbindin $\text{D}_{9\text{K}}$ are both upregulated by calcitriol in the rat (39), mouse (40), and chick (41). The calbindin proteins are under transcriptional regulation by calcitriol in animals (42), but posttranscriptional regulation appears to be quantitatively more important in humans (43). Calcium is capable of regulating calbindin $\text{D}_{28\text{K}}$ expression in chick kidney (44).

Regulation by parathyroid hormone (PTH) occurs predominantly in the distal tubule (17). One isoform of NCX (NCX1) appears to be the primary mechanism by which PTH modulates renal calcium reabsorption (45). The role of phospholipase D and protein kinase C and their effects on PTH-induced calcium reabsorption in the distal kidney tubule have recently been recognised (46). Interactions between vitamin D and PTH mediated effects on calcium transport have been reported in mouse distal kidney tubule cells (47). One report suggested that PTH may increase calbindin $\text{D}_{28\text{K}}$ expression in rats (48).

Estrogen increases calbindin $\text{D}_{28\text{K}}$ expression in rat kidney indicating the possibility of a common mechanism of hormonal regulation of calcium transport in the kidney (49). Recent studies in our laboratory reveal that distal kidney tubule PMCA is directly regulated by estrogen and testosterone in vitro (5).

THE ROLE OF THE INTESTINE IN EXTRACELLULAR CALCIUM BALANCE

The Physiology of Calcium Handling in the Intestine

In the adult human, 40–90% of the calcium consumed each day is excreted in feces and 10–60% is absorbed by the intestine. Three processes contribute to the overall role of the intestine in calcium balance: dietary calcium consumption, intestinal secretions

containing calcium and intestinal calcium absorption. Calcium enters the bowel not only from dietary sources but also from pancreatic, biliary and enterocytic secretions into the intestine. Under conditions of low calcium intake it is possible to excrete more calcium in the faeces from pancreatic, biliary and enterocytic secretions than is consumed in the diet. There are several known determinants of intestinal calcium secretion. In women these include dietary calcium intake (50) and possibly dietary phosphate intake (51). In rats oophorectomy affects calcium secretion (52). The site of action of these determinants or the effects of calciotropic hormones on intestinal calcium secretion is unknown. Net calcium absorption is the difference between the net amount of calcium consumed and the amount excreted in the feces. True calcium absorption takes into account the amount of calcium secreted into the intestine.

Dietary Factors Affecting Intestinal Calcium Absorption

Gut calcium absorption is determined by the intraluminal concentration of calcium achieved at various points in the bowel and by gut wall factors that affect absorption efficiency (e.g., vitamin D status). The actual site of calcium absorption in the bowel varies depending on the magnitude of the calcium load in the food and on its rate of transit through the bowel. In general, 95% of calcium absorption occurs in the small bowel. Although the duodenum has the highest rate of active absorption, it is not the most important site for calcium absorption on a quantitative basis except at very low calcium intakes. This is because the time that calcium resides within the duodenum is relatively short. In normal subjects lactose increases calcium absorption from 22 to 36% (53). However in patients with lactose intolerance, lactose will itself induce a reduction in calcium absorption of about 5% (53). This may be due to the osmotic effects of the lactose that reduces the effective concentration of calcium within the bowel (54). The connection between lactose intolerance and osteoporotic fracture would appear to be due primarily to a reduced calcium intake associated with avoidance of milk products (55).

High fibre diets have been recommended for various benefits on bowel and cardiovascular systems. Studies that have examined the effects of these diets on calcium consumption have not found deleterious effects, at least at moderate consumption of fibre containing foods (56). However at high fibre intakes, calcium retention is reduced from 25 to 19% (57).

Several studies have examined absorption in relation to the anion that accompanies dietary calcium (58,59). In general, the anion does not make much difference provided the calcium is well dissolved in the bowel lumen (60,61). It has been shown that in achlorhydric individuals the absorption of calcium carbonate is less than that of calcium citrate. This differential absorption is abolished if the calcium is taken with food (50). Calcium lactate gluconate has identical effects to milk powder that contains the same amount of calcium (62,63).

Mechanisms of Intestinal Calcium Absorption

The intestinal absorption of calcium occurs by transcellular and paracellular mechanisms. In general, the paracellular route is considered to be unregulated although there is some evidence that vitamin D can stimulate the nonsaturable phase of calcium transport (64). The driving forces behind the paracellular route of calcium uptake are thought to be the concentration gradient and solvent drag. Paracellular movement of calcium

takes place throughout the length of the intestine and may account for two thirds of calcium flux in the rat intestine. In the human, passive paracellular absorption appears to have an absorption efficiency of about 15%. Thus, at high dietary intakes, it may be possible to supply sufficient calcium to maintain extra cellular homeostasis from this source alone. Paracellular calcium secretion also occurs, primarily in the duodenum but also in the jejunum and ileum. Net calcium absorption is determined by the difference between paracellular absorption and secretion (66) balanced by transcellular absorption.

Cell Biology of Intestinal Epithelial Transport

There are two mechanisms of transcellular transport: active transport and transcellular vesicular transport termed transcalcitachia. Active transport involves both NCX1 and PMCA1b, the same two transporters present in the kidney distal tubule. They are expressed on the basolateral membrane of the enterocyte (67,68). NCX1 is active in the intestine in both rats (69) and humans (70) and appears to have less (approx 20%) calcium translocating activity than does PMCA1b in basolateral membrane preparations (69). This suggests that PMCA1b is the more important mechanism for calcium translocation in the intestine. The activity of PMCA1b in the rat declines with age (71). Calbindin D_{9K} but not calbindin D_{28K} is present within the proximal small intestine and exhibits a similar expression profile as PMCA1b (43).

Evidence for an endocytotic, exocytotic vesicular calcium transport mechanism also exists. Calcium transport into the cell may be increased by the opening of apical membrane calcium channels (such as ECaC) as the result of rapid, nongenomic stimulation by calcitriol, a process referred to as transcalcitachia (72). In this context, calcitriol stimulates calcium uptake into lysosomes at the apical membrane, with subsequent delivery to the basolateral membrane and a time course in the order of 30 min (73).

Hormonal Regulation of Intestinal Calcium Transport

PMCA1b activity and mRNA expression are stimulated by calcitriol (74). Calbindin D_{9K} expression is upregulated by calcium (75) and calcitriol in animals (76) and is correlated with serum calcitriol in humans (77,78). Posttranscriptional regulation of calbindin D_{9K} (79) appears to be quantitatively more important than transcriptional regulation (80).

Recent studies indicate that estrogen increases duodenal calcium transport in ovariectomized rats (independent of any effect on serum calcitriol), indicating another important role for estrogen in calcium transport across cell membranes (81). It is not clear which transport mechanism is affected by estrogen but estrogen has been shown to increase duodenal vitamin D receptor expression in ovariectomized rats (82). Thus estrogen and calcitriol may share an action on calcium transport in the intestine.

THE ROLE OF BONE IN EXTRACELLULAR CALCIUM BALANCE

The Physiology of Calcium Transport in the Basic Multicellular Unit

Bone structure can be divided into two types; trabecular and cortical bone. Each of these structures undergoes remodelling in which osteoclast-mediated bone resorption is followed by osteoblast mediated bone formation. The combination of the osteoclastic bone resorption and the consequent osteoblast mediated bone formation to refill the

resorbed area comprises the two components that define the basic multicellular unit (BMU) (83). This is one of the physiological bases for the maintenance of extracellular calcium homeostasis. The importance of osteoclast mediated bone resorption in the maintenance of extracellular calcium levels is evident from the dramatic effects of bisphosphonates, which directly inhibit osteoclast action. In Pagets disease of bone bisphosphonate therapy results in lowering of the ionised calcium concentration and consequent elevation of PTH and calcitriol (84). On the other hand, calcium reentry into the skeleton occurs at the time of new bone formation. The mechanism of extracellular calcification in the newly formed osteoid is still uncertain but may require the transport of calcium to the mineralizing area to allow the formation of hydroxyapatite. Thus calcium transport in the BMU consists of two major components, transport out of the bone under the influence of osteoclastic bone resorption and transport into the bone across the osteoblast to allow for the deposition of hydroxyapatite into matrix.

One of the principal advantages of osteoclastic-osteoblastic coupling in the BMU may be to allow for the local replacement of bone calcium by osteoclastic resorption lost during periods of low calcium intake. A phase of osteoblastic bone formation follows the phase of osteoclastic resorption, providing the opportunity to regenerate resorbed bone during a subsequent period of dietary calcium sufficiency. If the individual is in long term calcium balance, the amount of bone removed during low calcium intakes must be matched by the amount of bone replaced during adequate calcium intake. During episodes of calcium deprivation, there is a temporary imbalance in which bone resorption exceeds formation, thus releasing calcium into the circulation (85). During high calcium intakes, the bone deficit is replaced by a relative increase in bone deposition (86). This would constitute an elegant mechanism for smoothing out the demands on the skeleton for calcium during intermittent periods of dietary calcium deficiency without seriously impairing the mechanical function of the bone.

The activity of each BMU is regulated by PTH and calcitriol so as to play a role in the maintenance of extracellular calcium homeostasis. The regulation of the balance between osteoclast mediated release of calcium from bone and osteoblast mediated uptake of calcium into bone in relation to maintenance of extra cellular calcium homeostasis have not been elucidated (87). In addition to hormonal influences, there are likely to be site specific effects modulating the overall balance between bone and calcium accretion and dissolution, including mechanical influences and the presence of microdamage.

The Physiology of Calcium Transport Across Bone Lining Cells

In addition to the skeletal transport of calcium in relation to the BMU there is extensive histological and ultrastructural evidence for the presence of a bone lining cell layer or membrane made up of cells which probably arise from the osteoblast lineage (88). The function of this layer is uncertain but it may contribute to the maintenance of extracellular calcium homeostasis. The basis for this postulate is derived from calcium⁴⁵ studies combined with histological evaluations (89).

The activity of this lining cell layer may be modified by hormonal regulators involved in extracellular calcium homeostasis, including PTH and calcitonin (90–92). For example, in adult rats and humans bone formation is stimulated by PTH on preformed trabecular surfaces without a preceding episode of resorption (93,94) and thus it is likely that regulated calcium transport occurs across the bone lining cell layer. It is very dif-

difficult to determine the relative importance of this process in relation to the activity of the calcium transport into and out of the BMU.

Cell Biology of Calcium Transport in Bone: Osteoclasts and Osteoblasts

The mechanism of osteoclast mediated bone resorption is initially dependent on the development of a low pH in the subosteoclastic lacuna to allow dissolution of hydroxyapatite. This results from the formation of hydrogen ions by carbonic anhydrase type II in the osteoclast cytoplasm and their transport into the sealed subosteoclastic lacuna (65). Transport of organic and inorganic bone breakdown products, including movement of calcium from the resorption surface to the extra cellular fluid, probably occurs by vesicular transcytosis across the cell (95). PMCA protein has been found within the osteoclast, raising the possibility that transcellular cytoplasmic transport may also occur (96).

There is evidence that osteoclast activity is directly inhibited by the high extracellular calcium concentrations present in areas of resorption, perhaps by the induction of osteoclast apoptosis (97). There is also evidence for direct regulation of osteoclast resorptive activity via a membrane ryanodine receptor (98,99). The classical calcium sensing receptor may be expressed in mature osteoclasts and osteoclast precursors (100,101). Since calcitriol and PTH/PTHrP receptors have not been found in mature osteoclasts it is unlikely that they directly influence osteoclast activity.

All four calcium transporters, NCX1, PMCA1b, calbindin D_{9K}, and calbindin D_{28K} are expressed within the osteoblast, consistent with the notion that the osteoblast plays a direct role in calcium transport in bone. NCX1 is localized to the basolateral membrane of the osteoblast (102) and is downregulated by calcitriol and PTH (103). Whether NCX1 expression and activity is influenced by other endocrine/paracrine factors is unknown. Calbindin D_{28K} and calbindin D_{9K} have been identified in rat osteoblasts (104). Calbindin D_{28K} appears to be upregulated by calcitriol in human osteoblasts (105) and in chick bone (106). Calbindin D_{28K} suppresses apoptosis of murine primary osteoblasts, implicating this transporter in other cellular processes such as calcium buffering (107).

In contrast to the intestine and kidney distal tubule, PMCA1b is expressed on the osteoblast apical plasma membrane (108) and not on the basolateral membrane. The implications of this localisation in bone are not understood. In accord with the kidney distal tubule and intestinal enterocyte, PMCA1b is upregulated by calcitriol in osteoblast like cells (ROS 17/2.8) (109). This effect is not likely to be transcriptionally mediated (110). All these data indicate a role for the osteoblast in calcium homeostasis, that may be independent of its role in bone remodeling.

SIMILARITIES OF CALCIUM TRANSPORT MECHANISMS EMPLOYED BY CALCIUM TRANSPORTING EPITHELIA AND BONE MESENCHYMAL CELLS

The similarities between the regulation of calcium transporters in the kidney, intestine and osteoblast/bone lining cells are difficult to ignore and are exemplified by the effect of calcitriol on calcium transport. PMCA1b is responsive to calcitriol, an effect that appears to be hormone-specific and present in all three sites involved in calcium homeostasis. The PMCA1b isoform may, in fact, subserve two different physiological roles: The maintenance of intracellular Ca²⁺ concentration and the transcellular transport of Ca²⁺. These roles are unlikely to be mutually exclusive and could provide for diverse

physiological needs of a cell. PMCA1b may allow transcellular Ca^{2+} movement as well as the fine regulation of intracellular Ca^{2+} concentration. Both of these functions appear to be mediated efficiently and effectively by the PMCA1b isoenzyme in response to calcitriol. Localization of PMCA1b protein to the apical plasma membrane in osteoblasts and basolateral plasma membrane in kidney distal tubule cells and small intestinal enterocytes, suggest that upregulation of PMCA1b activity by calcitriol is likely to mediate an increase in renal tubular Ca^{2+} reabsorption, an increase in intestinal absorption and Ca^{2+} transport away from the mineralising surface of bone (109). Thus, the effects of calcitriol on PMCA1b play a dominant role in the maintenance of calcium homeostasis at the potential expense of bone matrix calcification. This concept may have important implications for the understanding of the regulation of bone mineral accretion and the maintenance of Ca^{2+} homeostasis.

PMCA1b and NCX1, are expressed on the same plasma membrane in the kidney distal tubule and small intestinal enterocyte but on opposing membrane surfaces in the osteoblast. This may be important as unlike the kidney distal tubule and small intestine, where calcium movement is unidirectional towards the ECF, the osteoblast may have to mediate bi-directional calcium fluxes. There may also be a need for the osteoblast to respond more quickly to changes in ECF calcium concentration and the ability of calcitriol to inhibit NCX1 activity and expression in the osteoblast basal membrane while stimulating calbindin $\text{D}_{28\text{K}}$ and PMCA1 at the opposing apical cell membrane could allow a rapid transcellular calcium delivery to the ECF space. In the kidney distal tubule PMCA1 and calbindin $\text{D}_{28\text{K}}$ are stimulated by calcitriol in a similar fashion but there is no corresponding effect to inhibit calcium export at the opposing plasma membrane since NCX1 is not expressed at this site. Consequently, the delivery to the basolateral membrane of calcium in the distal kidney tubule and small intestine epithelial cell is dependent only on the magnitude of calcium entry through apical membrane channels. This passive process of calcium entry into the epithelial distal kidney tubule and small intestinal enterocyte cell may not be as dynamic nor as versatile as the regulatory mechanisms employed within the osteoblast.

A detailed understanding of the effect of calcitriol on each Ca^{2+} transporter (PMCA1b, NCX1, calbindin $\text{D}_{28\text{K}}$, and calbindin $\text{D}_{9\text{K}}$) and how this relates to the functional effect of this and other hormones on transmembrane calcium transport is still not available. However, it is probable that the effects of hormonal agents on calcium transporters occur in a synchronized fashion. Calcitriol effects on PMCA1b, calbindin $\text{D}_{28\text{K}}$ and $\text{D}_{9\text{K}}$ hint at a common regulatory mechanism in each tissue. Recent data from the calbindin $\text{D}_{28\text{K}}$ knockout model have stimulated interest in the entire area of calcium homeostasis. Whilst hypercalciuria develops in the calbindin $\text{D}_{28\text{K}}$ knockout mouse (111), circulating serum calcium levels are maintained (112). What compensatory mechanism is operative in bone or intestine to account for this newfound equilibrium has yet to be explained (112).

Causation of Bone Loss in Osteoporosis

There are a large number of causes of bone loss. In general terms, they all result in negative whole body calcium balance. There is now good evidence that the bone loss occurring in women after the age of 65 is usually due to defects in intestinal calcium absorption and renal calcium reabsorption, in turn leading to increased resorption of bone calcium. However if the primary cause of the bone loss is due to mechanisms

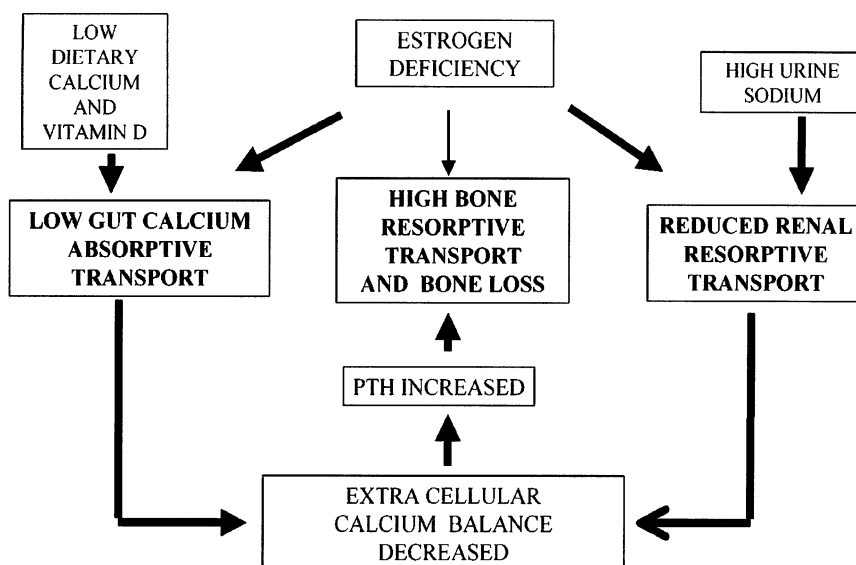


Fig. 4. The role of calcium transport in the causation of late postmenopausal bone loss.

originating in the skeleton itself (e.g., immobilization) attempts to augment extracellular calcium balance may only result in hypercalcaemia. Thus treatment with calcium is only of value where a reduction in calcium absorption in the intestine and reabsorption in the kidney is a cause of bone loss rather than a result of bone loss.

Age-Related Osteoporosis in Women: The Importance of Estrogen and Calcitriol Deficiency

An outline of the physiological interactions important in the development of negative calcium balance in aging women is shown in Fig. 4. In essence, osteoporosis can be regarded as a bihormonal deficiency disorder in which the importance of estrogen deficiency is most marked close to the menopause and in which the calcitriol deficiency becomes more important as renal function declines in later life. Principle causes of the decreased gastrointestinal absorption of calcium with aging (113,114) are decreased effects of calcitriol and estrogen effects on the gut. In addition, there may be an intrinsic age-related defect in the gut wall. The principle cause of the decreased reabsorption of calcium in the kidney is due to estrogen deficiency (4,115). The combined problem of reduced intestinal calcium absorption and decreased renal calcium reabsorption result in a negative extracellular calcium balance that results in increased bone resorption to maintain the calcium concentration in part by an increase in PTH (116–118). The increased bone resorption results in trabecular plate perforation and endocortical bone resorption. Another result of estrogen deficiency appears to be the release of calcium from specific skeletal sites, particularly those with trabecular bone. From a teleological perspective, the physiological connection between estrogen deficiency and skeletal calcium mobilization may relate to the need to supply calcium for lactation (119). Parallels with avian species are appropriate in that medullary bone formation, a prominent source of calcium supply for the eggshell, is thought to be dependent on estrogen. The avian osteoclast has estrogen receptors which when activated may reduce osteoclast activity (120).

Dietary Factors

As gastrointestinal calcium absorption and renal calcium reabsorption decline with age, dietary factors become important. For example, a low calcium intake exacerbates the intestinal calcium absorptive defect. A high salt intake exacerbates the renal calcium loss. Vitamin D deficiency also can exacerbate the calcitriol deficiency and impair gut calcium absorption.

Osteoblast Defect

An important factor in the development of age-related osteoporosis is a defect in osteoblastic bone formation that occurs with ageing. In the absence of adequate osteoblast activity the usual temporary effects of calcium deprivation on the skeleton are converted into permanent bone loss. This may account for the sensitivity of the aging skeleton to calcium deprivation. The causation of this age-related osteoblast defect has not been elucidated. Possibilities include reduction of osteoblast activation by decreases in physical activity and the stress strain effects activity induces at the bone surface. In animal experiments it is clear that mechanical effects stimulate periosteal bone formation (121). It is possible that the strain-related increase in bone density in postmenopausal women (122) is due to stimulation of osteoblastic activity. Another potential mechanism for the osteoblast defect is the reduction in IGF1 that occurs with aging (123). A further possibility relates to the potential anabolic effects of estrogen on the bone (124). Finally, there may be an intrinsic cell senescence mechanism perhaps associated with impaired generation of osteoblast precursors.

CONCLUSIONS

The basic mechanisms by which improvements in calcium balance prevent bone loss and fracture are beginning to be elucidated. Cell biological and animal experiments indicate the importance of calcium transport and highlight the significance of hormonal regulation of calcium transport proteins. Few human studies on the hormonal regulation of calcium transport proteins have been conducted. However, in vitro and animal studies support the concept, and help explain why calcium supplementation and hormonal therapy may reduce fracture risk primarily via effects on calcium homeostasis. It seems appropriate to recommend calcium supplementation to patients at risk of fracture.

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Calcium, Bone, and Life

Robert P. Heaney, MD

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CALCIUM AND THE ORIGINS OF LIFE ON EARTH

Calcium in the Biosphere

Calcium is the fifth most abundant element in the biosphere (after iron, aluminum, silicon, and oxygen). It is the stuff of limestone and marble, coral and pearls, seashells and eggshells, antlers and bones. Because calcium salts exhibit intermediate solubility, calcium is found both in solid form (rocks) and in solution. It was probably present in abundance in the watery environment in which life first appeared. Today, seawater contains approx 10 mmol calcium per liter (approx eight times higher than the calcium concentration in the extracellular water of higher vertebrates). Even fresh waters, if they support an abundant biota, typically contain calcium at concentrations of 1–2 mmol. In most soils, calcium exists as an exchangeable cation in the soil colloids. It is taken up by plants, whose parts typically contain from 0.1 to as much as 8% calcium. Generally, calcium concentrations are highest in the leaves, lower in the stems and roots, and lowest in the seeds.

Calcium-Protein Complexation and Life

Evolving life developed an intimate association with calcium, which it used both in the functioning of the most fundamental of cell processes and for the coordination of the myriads of cells and tissues that go to make up a complex organism. The calcium ion [Ca^{2+}] has an ionic radius of 0.99 Å, and is able to form coordination bonds with up to 12 oxygen atoms (1). The combination of these two features makes calcium nearly unique among all cations in its ability to fit neatly into the folds of the peptide chain. By binding with the oxygen atoms of glutamic and aspartic acid residues projecting off of the peptide backbone, calcium stiffens the protein molecule and fixes its tertiary structure. Magnesium and strontium, which are chemically similar to calcium in the test tube, have different ionic radii, and do not bond so well with protein. Lead and cadmium ions,

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by contrast, substitute quite well for calcium. In fact, lead binds to various calcium-binding proteins with greater avidity than does calcium itself. (This property is probably the principal basis for lead toxicity.)

Binding of calcium to various cell proteins results in activation of their unique functions (2). These proteins range from those involved with cell movement and muscle contraction to, for example, nerve transmission, glandular secretion, and cell division. In most of these situations, calcium acts both as a signal transmitter from the outside of the cell to the inside and an activator of the functional proteins within. In fact, ionized calcium is the most common signal transmitter in all of biology, operating in cells from bacteria all the way up to the highly specialized tissues in the higher mammals.

Intracellular Calcium and its Regulation

If all of the functional proteins of a cell were fully activated by calcium at the same time, the cell would rapidly self-destruct. For that reason, cells must keep free calcium ion concentrations in the cytosol at extremely low levels, typically on the order of $0.1 \mu\text{mol}$. This is 10,000-fold lower than the concentration of calcium ion ($[\text{Ca}^{2+}]$) in the extracellular water outside of the cell. Cells maintain this concentration gradient by a combination of mechanisms: (a) a cell membrane with limited calcium permeability; (b) ion pumps which move calcium rapidly out of the cytosol, either to the outside of the cell or into storage vesicles within the cell; and (c) a series of specialized proteins in the storage vesicles which have no catalytic function in their own right, but which serve only to bind (and hence sequester) large quantities of calcium. Low cytosolic $[\text{Ca}^{2+}]$ ensures that the various functional proteins will remain dormant until the cell activates certain of them; and it does this simply by letting $[\text{Ca}^{2+}]$ rise in critical cytosolic compartments.

CALCIUM IN THE HUMAN BODY

In land-living mammals, calcium accounts for 2–4% of gross body weight. A 60 kg adult human female typically contains about 1000–1200 g (25–30 mol) of calcium in her body. More than 99% of that total is in the bones and teeth. About 1 g is in the plasma and extracellular fluid (ECF) bathing the cells, and 6–8 g in the tissues themselves (mostly sequestered in calcium storage vesicles inside of cells).

In the circulating blood, calcium concentration is typically 2.25–2.5 mmol. 40–45% of this quantity is bound to plasma proteins, about 8–10% is complexed with ions such as citrate, and 45–50% is dissociated as free ions. In the ECF outside of the blood vessels, total calcium is on the order of 1.25 mmol. It is the ionic calcium concentration ($[\text{Ca}^{2+}]$) in the ECF which the cells see, and which is tightly regulated by the parathyroid, calcitonin, and vitamin D hormonal control systems.

ECF $[\text{Ca}^{2+}]$ is one of nature's great physiological constants, extending across the vertebrate phylum (at least in healthy individuals of the species concerned). When elevations of serum calcium occur in different physiological situations (such as during egg laying in reptiles and birds), the elevation is always in the protein-bound fraction, not in the ionized calcium concentration.

The ECF calcium serves two major groups of functions. It is the source of the calcium that pours into the cells of many tissues at the point of their activation, thereby triggering the specific cascade that produces tissue-specific cellular responses. Here, concentration is critically important, and clinicians have long recognized that hyper- and hypocalcemia are each associated with neuromuscular symptoms such as hypo- and hypertonia, con-

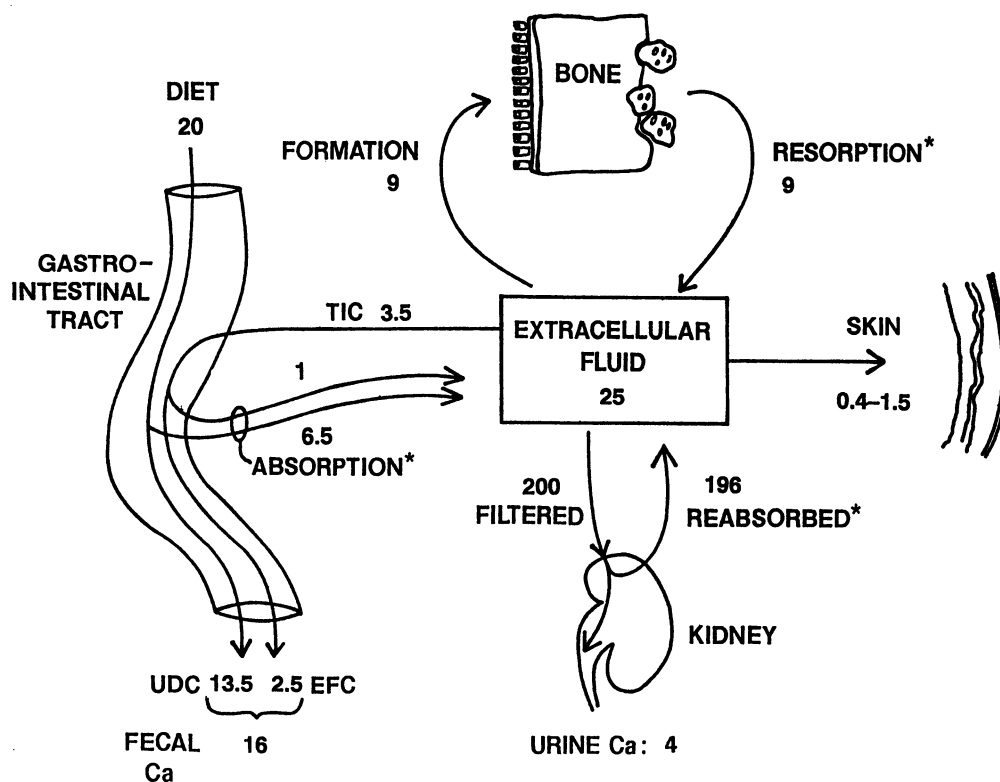


Fig. 1. Principal routes of calcium entry into and exit from the extracellular fluid of an adult human. Rates are given in mmol/d and represent typical values. (TIC is total intestinal calcium from endogenous sources, UDC is unabsorbed dietary calcium, and EFC is endogenous fecal calcium.) (Copyright Robert P. Heaney, 1996, used with permission.)

duction defects on electrocardiograms, and overt clinical symptoms such as constipation or muscular spasms and rigidity.

Calcium Traffic

The second role of ECF calcium is that its ions constitute the multidirectional calcium “traffic,” i.e., calcium entering the circulation through absorption of dietary calcium or resorption of bone calcium, and calcium leaving the blood in the process of bone mineralization, or through excretory or dermal losses. Both sets of processes are closely integrated in many complex ways, one of the more obvious of which is the fact that the physiological apparatus regulating ECF $[Ca^{2+}]$ also affects the fluxes in and out of the extracellular fluid.

Figure 1 depicts the principal routes of entry into and exit from the extracellular fluid in a healthy adult, and includes typical values for transfer rates. It is necessary to stress, however, that the indicated values of these transfer rates are highly interdependent. The individual processes will be considered briefly in the paragraphs that follow, but their interrelationships can be briefly summarized with an example. When absorptive input from the diet falls, bony resorption rises to offset the absorptive shortfall. This effect, as noted above, is produced by an increased secretion of parathyroid hormone. The immediate consequences are maintenance of the extracellular $[Ca^{2+}]$ and an offsetting reduction (however small) of the bony reserves of calcium.

Similarly, vigorous physical exercise leads to sweat losses that can be 10–20× the level of resting losses shown in Fig. 1 (4). Also, various nutrient-nutrient interactions may alter either calcium absorption efficiency or obligatory urinary calcium losses. Protein, for example, increases urinary calcium by about 0.025 mmol for every 1 g of protein ingested (5,6), and sodium (in the form of sodium chloride) increases urinary calcium by about 1 mmol per 100 mmol salt (7,8). These nutrient influences, together with great variability in food choices, and hence dietary calcium intake, constitute unregulated stresses on the system, i.e., they are perturbations to which the control mechanisms must respond. For all such stresses, bone resorption is promptly regulated up or down to compensate.

The examples just cited represent influences that, if not countered, would result in a lowering of ECF $[Ca^{2+}]$. But the opposite stress, i.e., a trend toward hypercalcemia, can be equally important and/or threatening. This half of the regulatory control environment is relatively uncommonly encountered in adult human physiology, largely because contemporary diets are low in calcium, and hypercalcemic stresses, accordingly, uncommon. However, animals with naturally high calcium intakes, subjected to thyro-parathyroidectomy but given thyroid replacement (i.e., deprived only of PTH and calcitonin) tend to exhibit not so much *hypocalcemia* as wildly fluctuating levels of ECF calcium, sometimes low, sometimes high, depending almost totally on absorptive inputs from the gut.

These examples are intended simply to illustrate the “push-pull” character of the regulatory system and the way it responds to unregulated inputs.

Dystrophic Calcification

With advancing age humans commonly accumulate calcium deposits in various damaged tissues, such as atherosclerotic plaques in arteries, healed granulomas, and other scars left by disease or injury, and often in the rib cartilages as well. These deposits are called dystrophic calcification and rarely amount to more than a few grams of calcium. These deposits are not caused by diet calcium but by local injury, coupled with the common tendency of proteins to bind calcium. So long as ECF $[Ca^{2+}]$ remains normal, calcification in tissues other than bones and teeth is a sign of tissue damage and cell death, not of calcium excess.

Bone as the Nutrient Reserve for Calcium

Aside from its obvious structural role, the skeleton is an important reservoir of calcium which serves to maintain plasma calcium concentrations. It does this principally by adjusting the balance between bone formation and bone resorption. An excess of the latter releases calcium into the blood, and an excess of the former soaks up calcium from the blood.

Additionally, this process of formation and resorption is what constitutes bone structural remodeling, or turnover. Remodeling of bone continues throughout life, and skeletal tissue is replaced every 10–12 yr on average. Bone-resorbing osteoclasts begin the remodeling process by attaching onto a bone surface, sealing it from the rest of the ECF; they then extrude packets of citric, lactic, and carbonic acids to dissolve bone mineral, and proteolytic enzymes to digest organic matrix. Later bone-forming osteoblasts synthesize new bone to replace previously resorbed bone.

Formation and resorption are coupled both systemically and locally, and when resorption is high, formation is generally high as well. But the coupling is not perfect. Bone

formation exceeds resorption during growth, and resorption exceeds formation during development of osteoporosis or in the face of ongoing dietary shortage of calcium. It is important to stress that calcium cannot be withdrawn from bone *per se*; instead it is scavenged from the tearing down of structural bony units. Thus, reduction in skeletal calcium reserves is equivalent to reduction in bone mass.

THE CALCIUM ECONOMY OF THE HUMAN ORGANISM: INPUTS, OUTPUTS, AND THEIR CONTROLS

Control Mechanisms

The concentration of calcium in the ECF is maintained by a combination of adjustments to the inputs and outputs in Fig. 1 and, perhaps most importantly, by controlling the level of the renal calcium threshold. This latter function, though very well established, is commonly underappreciated. Since that threshold is the point at which blood calcium begins to spill into the urine, it is clear that raising that point is a first line defense against renal calcium loss. Parathyroid hormone is the principal regulator of the renal calcium threshold. The importance of the threshold in the regulation of ECF $[Ca^{2+}]$ is clearly evidenced in the common clinical experience of the difficulty of elevating serum calcium in patients with hypoparathyroidism, even with sometimes heroic inputs of calcium into the system.

The physiological effects of PTH are complex and are diagrammed schematically in Fig. 2. These hormonal actions, in approximately the order in which they occur, can be described briefly as follows: 1) decreased renal tubular reabsorption of serum inorganic phosphate (P_i); 2) increased resorptive efficiency of osteoclasts already working on bone surfaces; 3) increased renal 1- α -hydroxylation of circulating 25(OH)-vitamin D to produce the chemically most active form of vitamin D; 4) increased renal tubular reabsorption of calcium; and 5) activation of new bone remodeling loci. These effects interact and reinforce one another in important ways, indicated by the connections between the loops of Fig. 2. For example, the reduced ECF P_i caused by the immediate fall in tubular reabsorption of phosphate is a potent stimulus to the synthesis of 1,25(OH) $_2$ D, and it also increases the resorptive efficiency of osteoclasts already in place and working in bone. 1,25(OH) $_2$ D directly increases intestinal absorption of both ingested calcium and the endogenous calcium contained in the digestive secretions. It also is necessary for the full expression of PTH effects in bone, particularly the maturation of cells in the myelomonocytic line that produce new osteoclasts.

The three arms of Fig. 2 make graphic the fact that the system uses three independent end-organs to regulate ECF $[Ca^{2+}]$; their actions are to reduce losses through the kidneys, to improve utilization of dietary calcium, and to draw down calcium from the bony reserves. The aggregate effect of them all, as Fig. 2 indicates, is to prevent or reverse a fall in ECF $[Ca^{2+}]$.

While hypocalcemia is a much more common risk in adults than is hypercalcemia, in infants and small children, both deviations are a threat. The principal defense against hypercalcemia is release of calcitonin by the C cells of the thyroid gland. Calcitonin (CT) is a peptide hormone with binding sites in the kidney, bone, and central nervous system. Absorption of calcium from an 8-oz feeding in a 6-month-old infant dumps 150–220 mg calcium into the ECF. This is enough, given the small size of the ECF compartment at that age (1.5–2 L), to produce near fatal hypercalcemia if other adjustments are not made.

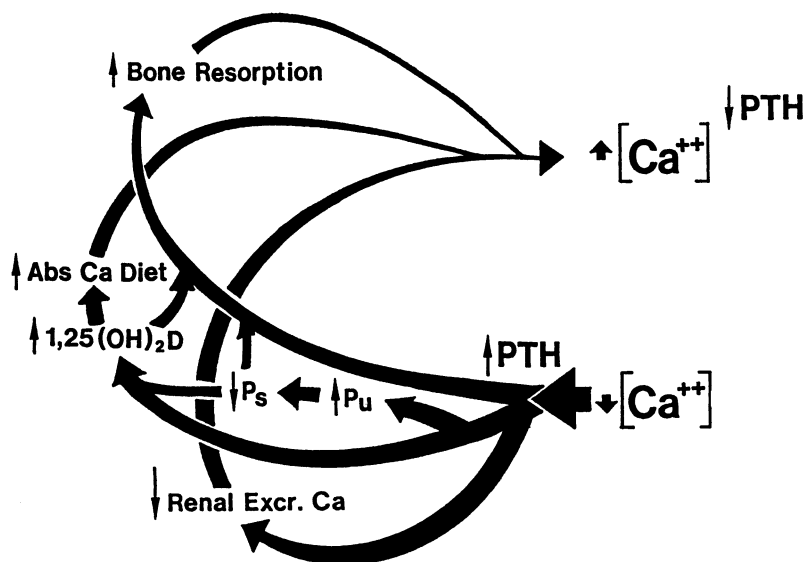


Fig. 2. Schematic depiction of the 3-arm control loop regulating ECF $[Ca^{2+}]$, showing specifically the response to a drop in $[Ca^{2+}]$. (P_s is serum inorganic phosphorus and P_u is urinary phosphorus clearance.) (Adapted from ref. 8). Copyright Robert P. Heaney, 1981. Used with permission.)

What happens is that CT is released, in part in response to the rise in serum calcium concentration, but even before that, in response to gut hormones signaling the digestive activity that will lead to absorption. This burst of CT slows or halts osteoclastic resorption, thus stopping bony release of calcium. Later, when absorption falls, CT levels fall also, and osteoclastic resorption resumes.

By contrast, CT has little significance in adults because calcium absorption is less efficient to begin with, and the ECF is vastly larger. As a result, transient absorptive calcemia from a high calcium diet raises the ECF $[Ca^{2+}]$ by only a few percentage points (approx 1% for each 100 mg ingested at typical intakes).

Endogenous Fecal Calcium Loss

Calcium is contained in all of the digestive secretions, as well as in the mucosal cells themselves (which turn over about every five days). Together these sources account for entry of endogenous calcium into the gut amounting to about 0.05 mmol (2 mg)/kg/d, or in a typical middle-aged woman, about 3.5 mmol (140 mg)/d (9). Because absorption efficiency for calcium is low, and because some of the digestive juice calcium enters the lumen downstream of the sites of most active absorption, most of this endogenous calcium ends up in the feces and is generally designated “endogenous fecal calcium (EFCa).” The quantity entering the gut is not regulated by the hormones otherwise controlling the calcium economy, and the principal known influences are phosphorus intake (9) and mucosal mass. EFCa, in turn, is inversely related to absorption efficiency (and hence to calcium intake). It constitutes one of the unregulated drains on the calcium economy to which the control system must react. EFCa is measurable only by isotopic tracer methods and hence cannot be assessed clinically. Nevertheless, when it is measured, it is found to account for a somewhat greater share of the variability in total body calcium balance than does actual oral calcium intake.

Table 1
Distribution of 24-h Urinary Calcium Values
in Normal Middle-Aged Women^a

	Percentile	mmol(mg)/d	mmol(mg)/kg/d
Estrogen-replete			
	97.5	6.3(252)	0.104(4.15)
	95.0	5.4(215)	0.093(3.72)
	90.0	4.9(197)	0.081(3.23)
	50.0	2.9(116)	0.046(1.86)
	10.0	1.5(62)	0.024(0.99)
	5.0	1.3(53)	0.021(0.83)
	2.5	1.1(44)	0.017(0.67)
Estrogen-deprived			
	97.5	7.6(303)	0.126(5.05)
	95.0	6.6(264)	0.107(4.27)
	90.0	5.6(225)	0.091(3.66)
	50.0	3.3(134)	0.054(2.15)
	10.0	2.0(81)	0.028(1.12)
	5.0	1.4(55)	0.020(0.80)
	2.5	0.9(38)	0.014(0.56)

^aAdapted from ref. 11.

Urinary Loss

Calcium losses in the urine are dependent upon filtered load except during adolescence. During this period of rapid growth, at calcium intakes typically ingested, most of the absorbed calcium is diverted to bone growth and little spills into the urine.

Machinery for calcium transport, most extensively studied in intestinal epithelial cells, is also present in the nephrons of the kidney, but it is not known if it is functional there. The process is calcium load dependent, stimulated by PTH and 1,25(OH)₂D, and has a microvillar myosin I-calmodulin complex that could serve as a calcium transporter (10). Active transport occurs in the distal convoluted tubule against a concentration gradient. Renal calcium clearance is increased when PTH concentration in blood is low, thereby protecting against hypercalcemia when bone resorption is high for reasons other than homeostatic. Tubular reabsorption is determined to some extent by Na⁺ excretion. For every 100 mmol of sodium excreted, approx 0.5–1.5 mmol of calcium is pulled out with it in the urine (6,7).

Urine calcium rises with absorbed calcium intake, but the relationship is loose and depends strongly on the circulating level of PTH at the time. This alimentary rise is partly due to the small increase in blood calcium following absorption of ingested calcium, with a corresponding increase in the filtered load of calcium. Available data from healthy adults indicates that urinary calcium rises on dietary intake with a slope of about +0.045, meaning that, for every 10 mmol rise in intake, urine calcium rises by about 0.45 mmol. But there is much variability about this average figure and the range of normal is accordingly very broad. Table 1 sets forth observed ranges in healthy estrogen-replete and estrogen-deprived adult women, both as absolute values and as weight-adjusted values. The latter can be applied to men since the difference in urine calcium between the sexes is due principally to the generally greater weight of men.

Illustrative of the dependence of urine calcium on the settings of the calcium economy is the fact that the sum of endogenous fecal and urinary losses has a much smaller coefficient of variation than does either route alone (11). In other words, as EFCa rises, urine calcium tends to fall, and vice versa, reflecting, in this instance, reciprocal renal conservation in the face of varying digestive juice losses.

Dermal Loss

Calcium is contained in all cells, and organs such as the intestinal mucosa, which turns over approximately every five days, thereby constitute a loss to the body of the calcium those cells contain. The same is true with epidermis and skin appendages (hair and nails), all of which contain some calcium. This shedding thereby produces a steady calcium drain on the system. It is the sum total of these cell-related dermal calcium losses which is represented in Fig. 1 by the rough estimate of 0.4–1.5 mmol/d. Sweat losses have not been extensively studied, but such data as are available indicate that heavy physical exercise in a hot environment, leading to extensive sweating, can increase sweat losses to levels as high as 5–10 mmol/d. In one study of athletes, these losses were sufficient to produce a measurable decrease in bone mineral density (i.e., a detectable reduction of the nutrient calcium reserve) across a playing season, despite the relatively high dietary calcium intakes typical of varsity athletes (3). A randomized controlled trial of calcium supplementation in the same athletes showed that supplemental calcium, above that which could be provided by diet, was able to prevent this seasonal, exercise-related bone loss. This instance probably represents an extreme situation, but it illustrates nicely the function of bone as the body's nutrient calcium reserve, and also a point, to be discussed further below, that, given relatively inefficient dietary extraction of calcium, there are limits to how much calcium the organism can get from food to offset unregulated losses.

Intestinal Absorption

Intestinal calcium absorption occurs by two pathways: 1) transcellular, saturable (active) transfer that involves a vitamin D-dependent calcium-binding protein, calbindin; and 2) paracellular: a nonsaturable (diffusional) transfer that is to some extent a linear function of the calcium content of the chyme.

Active absorption is more efficient in the duodenum and proximal jejunum where calbindin is present in highest concentration. However, total absorption is probably greater in the ileum where the residence time is longer. Absorption from the colon accounts for about 5% of the total amount absorbed in normal individuals but may be larger in patients with small bowel resections and in individuals in whom colonic bacteria break down dietary calcium complexes.

The main regulator of transport across the epithelial cell against the energy gradient is $1,25(\text{OH})_2\text{D}$, which controls the synthesis of calbindin by DNA transcription upon binding of the hormone with receptors in the nucleus. Calbindin operates by binding Ca^{2+} on the surface of the cell, then internalizing the ions via endocytic vesicles that probably fuse with lysosomes. After release of the bound calcium in the acidic lysosomal interior, the calbindin returns to the cell surface, and the Ca^{2+} ions exit the cell via the basolateral membrane (12). Calbindin serves both as a Ca^{2+} translocator and a cytosolic Ca^{2+} buffer. Relative Ca^{2+} binding capacities across the enterocyte are brush border = 1, calbindin = 4, and the ATP-dependent Ca^{2+} pump = 10, a gradient that ensures unidirectional transfer of Ca^{2+} (15).

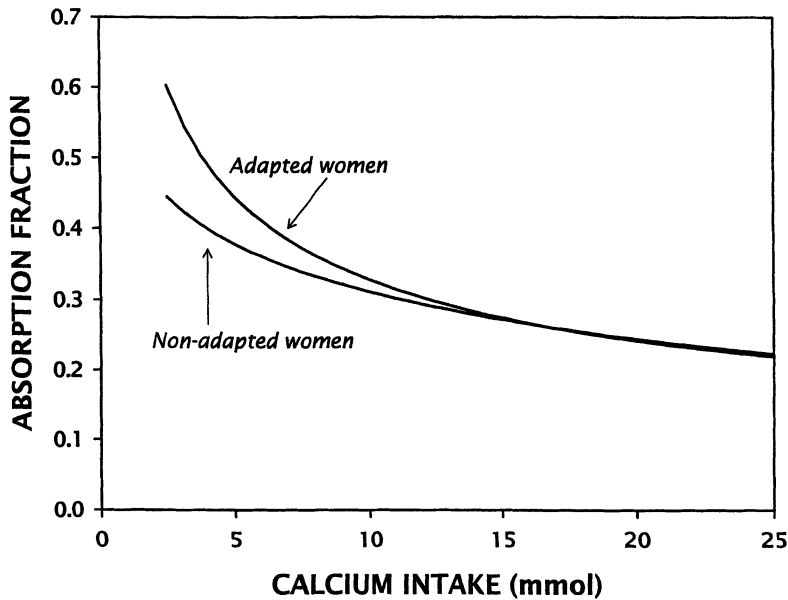


Fig. 3. Relationship between calcium intake and absorption fraction in women studied on their usual calcium intakes (adapted) and in women tested with no prior exposure to the test load (nonadapted). (Copyright Robert P. Heaney, 1999. Used with permission.)

In the paracellular pathway, calcium movement occurs not through the cell interior but through the junctions that bind one cell to another, i.e., *around* the cells. Theoretically, this can be in both directions, but normally the predominant direction is from lumen into blood. Rate of transfer depends on calcium load and tightness of the junctions. Water probably carries calcium through the junctions by solvent drag (14). Calcium usually is freed from complexes in the diet during digestion and is released in a soluble and probably ionized form for absorption. However, low-molecular-weight complexes, such as calcium oxalate and calcium carbonate, can be absorbed as non-dissociated compounds (15).

The relationship between calcium intake and absorption fraction is shown in Fig. 3. At lower calcium intakes, the active component contributes importantly to absorbed calcium. As calcium intakes increase, the active component becomes saturated and vitamin D-mediated synthesis of calbindin drops. Thus an increasing proportion of calcium is absorbed by passive diffusion. The figure illustrates that, across most of the intake range, the adaptive component is rather small. This partly explains the inefficiency of human ability to compensate for a fall in calcium intake.

Various host factors affect calcium absorption efficiency. Vitamin D status, intestinal transit time, mucosal mass and stage of life are the best established. In infancy, absorption is dominated by paracellular diffusion. For that reason, the vitamin D status of the mother has little effect on calcium absorption in young breast-fed infants. Both active and passive calcium transport are increased during pregnancy and lactation. Calbindin and plasma $1,25(\text{OH})_2\text{D}$ and PTH levels increase during pregnancy. From midlife on, absorption efficiency declines by about 0.2 absorption percentage points per year, with an additional 2% decrease at menopause (16).

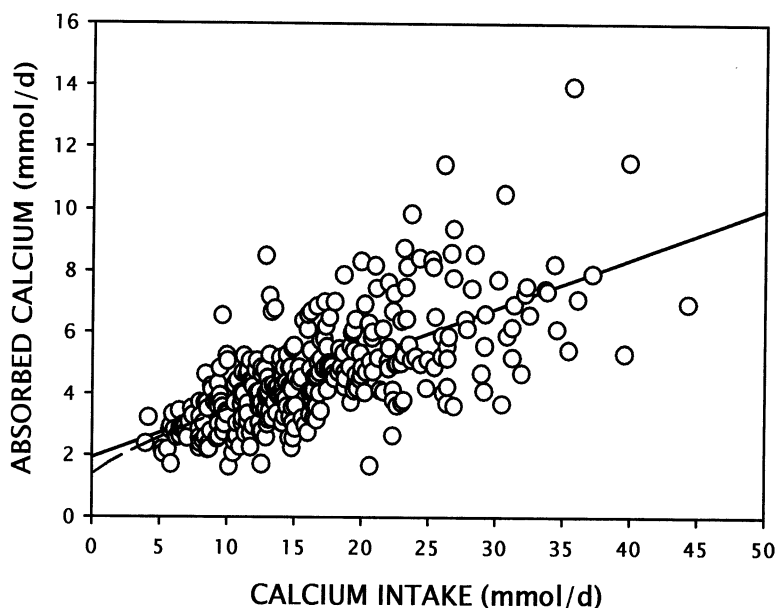


Fig. 4. Absorbed calcium plotted as a function of intake in 332 studies in middle-aged healthy women studied on their usual calcium intakes. (Copyright Robert P. Heaney, 2001. Used with permission.)

It has long been recognized that calcium absorption efficiency increases as the size of the ingested load falls. This relationship has two components, an effect of load itself and variation in vitamin D-mediated active absorption. Within individuals, absorptive efficiency generally varies approximately inversely with the logarithm of intake, but the *absolute* quantity of calcium absorbed increases nonlinearly with intake (17,18). However, only 20% of the variation in calcium absorption can be accounted for by differences in intake. Individuals seem to have preset absorptive efficiencies, some high, others low.

The canonical inverse relationship between intake and absorption fraction has often been uncritically assumed to mean that the body can adapt perfectly well to reduced intake. However, extensive studies in which absorption has been measured by isotopic tracer methods show very clearly that, while fractional absorption does rise (*see*, for example, Fig. 3), the rise is far short of what would be needed to maintain a constant mass transfer rate across the intestinal mucosa. Figure 4 illustrates this point with one such set of data. The regression line through the data in Fig. 4 is for a simple linear model, and more detailed investigations of the low intake end of the curve indicate that the rise is initially steeper, reflecting the active transport response to low intake discussed above. The slope of the line in Fig. 4 is +0.158, meaning that 15.8% of ingested calcium is absorbed, overall. If analysis is confined to intakes at the high end of the range, the slope drops to about +0.12. This means that the body absorbs from ~12% of any additional amount of calcium that may be ingested. At all intakes, the distribution of absorption values is broad, as the spread of the data in Fig. 4 demonstrates.

The relationship of absorption specifically to load size is illustrated in Fig. 5, which summarizes the data from three groups of sources: milk calcium (the principal dietary source of calcium in the industrialized nations), calcium carbonate (the principal calcium salt used in calcium supplements), and finally calcium oxalate. What the figure

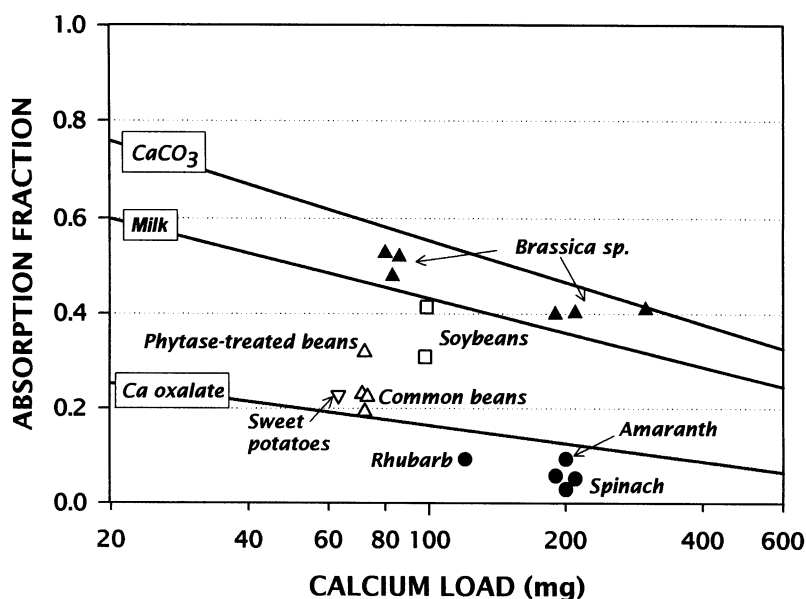


Fig. 5. Regression lines fitted to fractional absorption values at various load sizes for three families of calcium sources. Topmost is the line for plain calcium carbonate. Next is the line for milk calcium. The lowest is the line for calcium oxalate and the high oxalate vegetables (e.g., spinach and rhubarb). For all three groups there is an inverse linear relationship with the logarithm of load size, i.e., at low load sizes, a larger fraction of the load is absorbed than at high loads. Mean fractional absorption values for various other food sources are plotted for their respective intake loads. (Copyright Robert P. Heaney, 2001. Used with permission.)

clearly shows is that altogether apart from the intrinsic absorbability of the calcium source, absorption varies linearly and inversely with the logarithm of the load size. Furthermore, since all of the studies summarized in Fig. 5 were acute studies, in which the subjects were not given an opportunity to habituate themselves to a particular calcium source or level of calcium intake, the relationships to load depicted are purely physical, i.e., there is no physiological adjustment component, i.e., no compensating alteration of $1,25(\text{OH})_2\text{D}$ -mediated active absorption.

There are several practical consequences of this load relationship. One is that dividing calcium intake into multiple doses over the course of a day results in much more efficient absorption than ingesting the same total quantity in a single dose. This point is illustrated in an experiment shown in Fig. 6, in which healthy individuals were given the same tracer-labeled calcium load (25 mmol), either as a single bolus at breakfast, or as 17 individual doses of 1.47 mmol at half-hour intervals, starting with the same breakfast (19). Figure 6 shows graphically, and pharmacokinetic calculation reveals explicitly, that the area under the curve (AUC_∞) for the divided dose regimen was nearly twice that for the single dose regimen. A related consequence deals with the interpretation of published studies in which calcium supplements were used. Even if the aggregate daily doses were the same in two studies, when the dosing regimens are different, the effective delivered dose will be predictably different.

It is worth noting in passing that the primitive human diet, which would have been relatively calcium-rich in most of its constituents, would more closely have approxi-

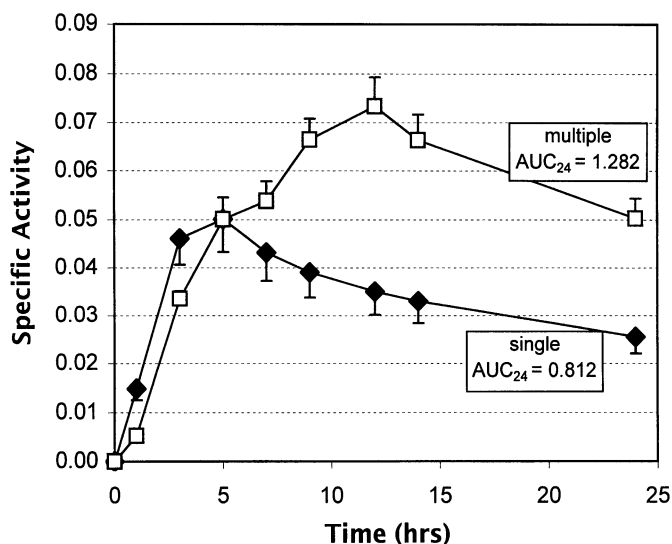


Fig. 6. Time course through 24 h for the mean specific activity values for two calcium dosing regimens. In the first (labeled “single”), 1000 mg Ca (25 mmol) was ingested as a single bolus at breakfast, and in the second (labeled “multiple”), the same total load was ingested in 17 equally spaced doses of 59 mg (1.5 mmol) each, ingested at 0.5-h intervals. (Copyright Robert P. Heaney, 2000. Used with permission.)

mated the continuous dosing regimen. Hence, not only would the primitive calcium intake have been higher than we currently experience, but its pattern of ingestion would likely have delivered calcium into the body more efficiently than modern humans generally manage.

Bone Calcium Turnover

As the numbers in Fig. 1 suggest, the turnover of bone, in the process of bone modeling and remodeling accounts for roughly half of the total turnover of the ECF in a typical healthy adult. (The proportion would be substantially higher during growth.) A single cubic centimeter of bone contains ~10 mmol calcium, equivalent to ~40% of the total calcium in the entire ECF of an adult. Essentially all of that bone calcium is locked away in intimate association with the collagen fibers of the bone matrix, and it can be released into the blood only by physically tearing down a unit of bone through osteoclastic resorption. Similarly, calcium deposition in bone occurs as a result of another cellular activity, the osteoblastic deposition of collagen matrix and its subsequent alteration to create crystal nuclei suitable for aggregating calcium and phosphate as hydroxyapatite. Both processes are cell-mediated. However, with mineral deposition, the timing of the mineral entry lags behind the cell's deposition and activation of the matrix. Since hormonal control mechanisms, whether endocrine or paracrine, act only through functioning cells, it follows that mineral deposition in bone is much less *acutely* controllable than is mineral removal. Previously nucleated bone matrix creates a mineral drain, or debt, which is paid by extracting mineral from blood flowing past the new bone-forming site, and stopping osteoblastic bone formation will not stop mineralization of the last several days' accumulation of deposited matrix. By contrast, both PTH and calcitonin, and their attendant mechanisms can act very promptly on osteoclastic resorption. Hence it is the

resorptive component of bone turnover which is the one most responsive, in the scheme of Fig. 2, to alterations of input into and output from the body.

Quantitative Operation of the System

Although the operation of the calcium regulatory system, or any feedback loop for that matter, must first be sketched out *qualitatively* (as in Fig. 2), in the final analysis it is the *quantitative* operation of the system that will determine what ultimately happens (for example, to the size of the calcium reserve, i.e., the mass of the skeleton). This *quantitative* working of the system for adjusting inputs and losses in response to dietary and other perturbations is often ignored. For example, it is commonly, if erroneously, assumed that, because intestinal calcium absorption efficiency varies inversely with intake, the body can fully compensate for declines in intake or increases in excretory loss. But quantitative analysis of the system (as well as data such as those assembled in Fig. 4) shows the fallacy of that assumption. In the face of reduced intake, ECF $[Ca^{2+}]$ tends to fall, and the prior rate of absorption of food calcium no longer suffices. The result is an increase in PTH secretion, which produces the three end-organ effects just described, i.e., more bone resorption, improved renal conservation, and increases calcium absorption efficiency. In brief, all three control loops are called upon to offset a shortfall caused by just one of them. The net effect with respect to total bone mass depends both on the relationship between the responsiveness of the three effector organs and on their capacity to provide the needed calcium (20). *Sensitivity* of the effectors is genetically and hormonally determined, whereas *capacity* to respond is largely determined by unregulated factors outside the control loop, such as the calcium content of the diet and factors that influence obligatory loss.

If for some reason the response of one or the other of these effectors is blunted, PTH rises further, forcing more response from the other two effectors. Conversely, if one effector (such as bone) is highly responsive to PTH, the hormone level rises less because the needed calcium is readily supplied from the nearly limitless skeletal reserves. As a result, less improvement in external calcium utilization ensues. Similarly, if the gut is unresponsive or the diet is so low in calcium that its capacity to yield the needed amount is exceeded, then PTH secretion rises further and bone is driven to meet the needs of the ECF $[Ca^{2+}]$. The two key insights here are (1) it is ECF $[Ca^{2+}]$ that is being regulated, not bone mass, and (2) the dose-response curves for the three effector systems are independent of one another.

Examples of different patterns of effector responsiveness abound. Thus, American blacks (and probably African blacks as well) have a bony resorptive apparatus relatively resistant to PTH (21–23). As a result, they develop and maintain a higher bone mass than do Caucasians and Orientals, despite an often poor diet. As predicted from the foregoing, African-Americans exhibit higher PTH and calcitriol levels, but lower levels of bone remodeling. In brief, they utilize and conserve diet calcium more efficiently than Caucasians. Somewhat the opposite situation occurs in all women at normal menopause. Because estrogen appears to decrease bony responsiveness to PTH, estrogen loss at menopause increases the skeletal response to PTH. This is a part of the explanation for the increase in recommended calcium intake after menopause (24,25). Obese individuals also increase their bone mass as they gain weight (26), and they lose less bone at menopause (27). Like blacks, they have high circulating PTH levels and (presumably) a relatively resistant bone remodeling apparatus.

Age-Related Changes in Operation of the Control System

Important changes occur in these quantitative settings of the system with age, as well as in unregulated inputs. An example of the latter is the fall in calcium intake among women in the United States from early adolescence to the end of life. In NHANES-II, median calcium intake was 793 mg (~20 mmol) in early adolescence, 550 mg (~14 mmol) in the 20s, and 474 mg (~12 mmol) at menopause (28). At the same time, absorption efficiency also falls with age. * Peripubertal girls absorb calcium with about 45% greater efficiency for the same intake than do perimenopausal women (29). As already noted, after age 40 yr, absorption efficiency drops by about 0.2 absorption percentage points per year, with an added 2.0 percentage point drop across menopause (16). In concrete terms, if a 40-yr-old woman absorbed a standard load at an efficiency of 30%, the same woman, at age 65 and deprived of estrogen, would absorb at an efficiency of 22.8%, or almost a 25% worsening in absorptive performance.

To complicate the situation further, renal calcium clearance rises at menopause (30), an effect seen most clearly with low calcium intakes, when urinary calcium will typically be as much as 36% higher than at premenopause (11). Vitamin D status declines with age as well (31,32), although this is also a function of declining solar exposure, dermal vitamin D synthetic efficiency, and milk consumption. In Europe, where solar vitamin D synthesis is low for reasons of latitude and climate, and milk is generally not fortified, serum 25(OH)D concentration drops from over 100 nmol/L (40 ng/mL) in young adults to under 40 nmol/L (16 ng/mL) in individuals over age 70 yr.

Not surprisingly, serum PTH rises with age as a consequence of this aggregate of age-related changes. 24-h integrated PTH is 70% higher in healthy 65-yr-old United States women consuming diets containing 800 mg Ca per day than in third-decade women on the same diets (33). That this difference is due to insufficient absorptive input is shown by the fact that the difference can be completely obliterated by increasing calcium intake (33).

Two Examples of System Operation

As stressed in the foregoing, it is a quantity that is being optimized (i.e., ECF $[Ca^{2+}]$); this is accomplished by the algebraic sum of various quantitative inputs and outputs. Two examples will serve to illustrate further the importance of attending to quantities. One examines the contrast between calcium handling at menarche and menopause just described, and the second describes the response of the system at any given age to a fixed increase in obligatory loss.

MENARCHE AND MENOPAUSE

True trabecular bone density increases by about 15% across menarche (34), and about the same quantum of bone is lost across menopause (35). Curiously, administration of estrogen to women more than 3 yr postmenopausal has generally failed to reproduce the pubertal increase in BMD, and it has been customary to say, in recent years, that apart from whatever remodeling transient estrogen (or hormone) replacement therapy (ERT/

*A part of this absorptive decline is due to estrogen deficiency, which both decreases renal 1- α -hydroxylation of 25(OH)D and appears to have a small effect on the intestinal mucosa. A further part may be due to decrease in mucosal mass, which, in animals varies with food intake.

Table 2
Net Calcium Absorption at Menarche and Menopause

	<i>Menarche</i>	<i>Menopause</i>
Ca intake ^a	793 mg/d (19.8 mmol/d)	474 mg/d (11.8 mmol/d)
Ca absorption efficiency ^b	35.2%	30.5%
Endogenous fecal Ca ^c	67 mg/d (1.7 mmol/d)	102 mg/d (2.5 mmol/d)
Net Ca absorption	212 mg/d (53 mmol/d)	42 mg/d (10.5 mmol/d)

^aNHANES-II median values (28).

^bHeaney et al. (16); O'Brien et al. (20); values adjusted to intake.

^cHeaney et al. (9).

HRT) may produce in postmenopausal women (36), the principal effect of ERT/HRT on bone is to stabilize bone mass, rather than to cause restoration of what had been lost. But this conclusion was drawn without attending to the quantitative aspects of the age-related changes in the calcium economy, summarized in the foregoing.

Table 2 assembles published data for median calcium intake and mean data for absorption efficiency and endogenous fecal calcium loss, and shows very clearly how quantitative changes occurring in the 40 yr from menarche to menopause account for the rather different performance of the two age groups. In brief (and despite an intake less than recommended), a peripubertal girl is able to achieve net absorption of over 200 mg (5 mmol) calcium from the median diet of her age cohort, whereas an early menopausal woman extracts less than one-fifth as much from hers. The drop in intake amounts to about 40%, but the fall in net absorption is 80%. As Table 2 shows, this is the resultant of lower intake, lower absorption efficiency, and higher digestive juice calcium losses. Given the level of total body obligatory losses at midlife, this absorbed quantity is simply not sufficient to support an estrogen-stimulated increase in BMD. As would be predicted from this understanding, higher calcium intakes permit estrogen to produce in postmenopausal women bony increases closer to those seen at puberty (37).

RESPONSE TO AUGMENTED LOSSES

As already noted, it is commonly (and uncritically) considered that the absorptive apparatus is able to compensate either for a change in intake or a change in excretory loss. However, quantitative considerations make it clear that this depends entirely on the level of calcium in the diet. Thus, an individual increasing his/her sodium intake by an amount equivalent to a single daily serving of a fast-food, fried chicken meal experiences an increase in urinary calcium of about 1 mmol (40 mg)/d. Without compensating adjustments in input to the ECF, $[Ca^{2+}]$ would drop. PTH, of course, rises, and with it, synthesis of $1,25(OH)_2D$, resulting ultimately in better extraction of calcium from the diet.

Published data allow rough estimation that a calcium drain of this magnitude produces an increase in $1,25(OH)_2D$ of about 6–7 pmol/L (38), and dose-response measurements for $1,25(OH)_2D$ indicate that this stimulus would increase calcium absorption efficiency by about 2–3 absorption percentage points (39). A 2–3% increase in extraction from a 50-mmol (2000 mg) diet yields 1–1.5 mmol (40–60 mg) of extra calcium, more than enough to offset the increased urinary loss, whereas, from a 5-mmol (200 mg) diet, the

same absorptive increase yields less than 0.1 mmol (4 mg).^{*} Thus, on a high-calcium diet, the body easily compensates for varying drains: both bone and ECF $[Ca^{2+}]$ are protected. But on a low-calcium diet, although the ECF $[Ca^{2+}]$ is protected, bone is not. Why does serum $1,25(OH)_2D$ not rise more on a low-calcium diet? Simply because the $1-\alpha$ -hydroxylation step is responding to PTH. Bone calcium meets much (or most) of the ECF need, and PTH secretion is regulated by ECF $[Ca^{2+}]$, not by bone mass.

In brief, as the body adjusts to varying demands, the portion of the demand met by bone will be determined both by factors influencing bony responsiveness and by the level of diet calcium, the principal component of the system that is not regulated. However, it must also be stressed that, although an adequate calcium intake is a necessary condition for bone building and for adaptation to varying calcium demands, it is not by itself sufficient. Calcium alone will not stop estrogen-deficiency bone loss nor disuse bone loss (because neither is due to calcium deficiency). But by the same token, recovery from immobilization or restoration of bone lost because of hormone deficiency will not be possible without an adequate supply of the raw materials needed to build bone substance.

THE CALCIUM REQUIREMENT

In adults the calcium requirement is the amount of dietary calcium required to replace losses through urine, feces, and skin. (During growth, pregnancy and lactation, the requirement includes as well the calcium needed for skeletal growth, fetal development, and milk production.) Calcium is a threshold nutrient. Above a certain intake, retention plateaus and little further increase in calcium retention occurs. The ingested excess is simply excreted. Threshold intakes for achieving maximal calcium retention were used to set the 1997 dietary reference intakes for calcium (25). Recommendations by various national policy groups for calcium intake across the life span are given in Table 3. Calcium requirements for bone health are not uniform throughout life first because of changes in skeletal growth and then because of changes related to age in absorption and excretion.

Childhood and Adolescence

Net calcium accretion continues from birth through the late twenties. Rate of growth slows from the infancy high to about age 8 yr, and then increases rapidly again. Maximal accretion occurs during the pubertal growth spurt, which occurs for most girls between the ages of 12 and 14 yr and for boys, between 14 and 16 yr. The intake required for mean maximal calcium retention in adolescents is 32.5–40 mmol 1300–1600 mg/d (41). Between the ages of 9 and 17 yr, approx 45% of the adult skeleton is acquired. Although not a linear process, this represents an average gain in bone mass of about 7–8%/yr.

Several calcium or dairy supplementation trials have been conducted in children. They all demonstrate that calcium intake can positively influence bone accumulation (42–45). However, as would be expected for any nutrient ingested basally in suboptimal amounts, the differential gain in bone mass wanes after cessation of calcium supplementation (45).

After adult height is achieved, calcium accretion continues during the phase of bone consolidation (which varies from one skeletal site to another, but for most is probably some time in the late 20s). At the end of consolidation, when the maximum amount of bone has been accumulated, the adult is said to have achieved his or her peak bone mass.

^{*}This is partly because extraction efficiency is already relatively high on low intakes, and there is less calcium still unabsorbed on which the mucosa can work to extract additional calcium.

Table 3
Various Estimates of the Calcium Requirement in Women^a

Age	1989 RDA ^b	NIH ^c	1997 DRI (AI) ^d
1–5	800	800	500/800
6–10	800	800–1200	800/1300
11–24	1200	1200–1500	1300/1000
Pregnancy/lactation	1200	1200–1500	1000
24–50/65	800	1000	1000/1200
65–	800	1500	1200

^aAll values are given in mg, as this is how the respective bodies reported their recommendations. To convert to SI units, divide the values in the table by 40.

^bRef. 40.

^cRecommendations for women as proposed by the Consensus Development Conference on Optimal Calcium Intake (24).

^d“AI” refers to “Adequate Intake” (25), a value which, in this context, is equivalent to an average requirement. The corresponding RDA could be 20–30% higher, i.e., 1000 in children, 1600 in adolescents, 1200 in adults out to age 50, 1200 during pregnancy and lactation, and 1450 in those over age 50. The presence of two values in this column reflects the fact that the age categories for the DRIs overlapped those of the NIH.

However, the timing of peak bone mass varies with the skeletal site. The hip achieves peak bone mass at approx age 17–18 yr, whereas the spine can add mass throughout most of the 3rd decade of life in females (26). The skull accumulates bone throughout life, as does the femur shaft (26,47).

Although 60–80% of peak bone mass is genetically predetermined, a number of environmental factors also importantly affect bone mass. The two are not mutually exclusive because genetic factors are often expressed in the way the organism responds to its environment. Aside from calcium intake, other lifestyle choices that affect peak bone mass include physical activity, intake of other nutrients that alter calcium utilization (covered elsewhere in this chapter), anorexia, and substance abuse. Beyond the age of peak bone mass, lifestyle choices can affect rate of bone loss, but the window of opportunity to build bone is closed some time before age 30.

Adults

The mature female body contains ~25–30 mol (1000–1200 g) calcium, and the mature male, ~30–40 mol (1200–1600 g). The population coefficient of variation around these means is about 15%. Total body bone mass remains relatively constant over the reproductive years, as decreases in the proximal femur and other sites after age 18 yr are offset by continued growth at the forearm, total spine, and head. Then, at mid life, menopausal and age-related bone loss sets in. Menopausal loss occurs most rapidly during the two years prior to and the three years following menopause, and amounts to 5.3% at the upper femur and 10.5% at the lumbar spine (48). The average older adult loses bone at a rate of 0.5–1%/yr, rising to as high as 3%/yr by age 80 yr (49). The explanation for bone loss during aging includes a variety of causes, such as declining calcium intakes, declining physical activity, decreased levels of gonadal hormones, decreased circulating levels of 1,25(OH)₂D, intestinal resistance to 1,25(OH)₂D, decreases in calcium absorption, and decreases in renal calcium conservation.

The threshold intake need to maintain this bone mass during the reproductive years is 20–25 mmol 800–1000 mg/d. After age 50–60 yr the threshold intake required to minimize age-related loss rises to 30–40 mmol 1200–1600 mg/d.

CALCIUM SOURCES

Dietary Considerations

Dietary sources and calcium intakes have changed considerably during human evolution. Early humans derived calcium from roots, tubers, nuts, and greens, as well as the bones of small prey, in quantities believed to exceed 37.5 mmol (1500 g) per day (50), and perhaps up to twice this amount when calculated on the basis of consuming food to meet the caloric demands of a hunter-gatherer of contemporary body size. After domestication of seed-bearing plants, calcium intakes decreased substantially because the staple foods became cereal grains and fruits, the plant parts that contain the least calcium. Consequently, the modern human often consumes insufficient calcium to optimize bone density. The food group that supplies the bulk of the calcium in the Western diet is now the dairy food group, which was not represented at all in the paleolithic diet.

Bioavailability

The term “bioavailability” is pharmacologic in origin, and has complex connotations. But for mineral ions, and particularly for calcium, these complexities reduce approximately to a simple matter of absorbability. Bioavailability can be measured in several ways, perhaps the most direct and straightforward being the introduction of a suitable isotopic tracer into the calcium source, and then the calibrated measurement of the appearance of that tracer in body fluids. These methods permit direct estimation of true unidirectional flux from the intestinal lumen into the body, usually expressed as a fraction of the ingested load. Load size, as discussed earlier, is itself an important determinant of fractional absorption; hence it is not possible to establish a single value for bioavailability for any given calcium source, since its absorption fraction will be dependent upon how much was consumed. Clearly, therefore, comparative studies require comparable loads.

A second approach is to measure the small rise in serum total calcium or ECF $[Ca^{2+}]$ following oral ingestion. As already discussed, the body attempts to damp out these absorptive rises; hence they tend to be small and correspondingly require very precise measurement of serum calcium (total or ionized). These methods do not permit estimation of fractional absorption, but can be useful in comparing two or more substances, and particularly substances into which it is not possible to introduce a suitable isotopic tracer.

Less sensitive measures include measurement of the increment in urine calcium that is the inevitable concomitant of the absorptive rise in serum calcium, or, alternatively, measurement of the decrement in serum PTH evoked by the rise in serum calcium. These are less sensitive both because they are inherently more variable (both within and between subjects), and because the assay methods introduce an additional level of imprecision in their own right.

In general, the isotopic tracer methods have the smallest sample size requirement, the increment in serum calcium, an intermediate requirement, and changes in urine calcium or PTH, the largest sample size. Finally, only the isotopic tracer methods produce results that directly translate to true fractional absorption.

Bioavailability is important in this context because not all calcium sources have equally available calcium. As discussed earlier (*see* Fig. 3), vegetable greens of the

mustard family have an intrinsic bioavailability slightly greater than that of milk, whereas other vegetables such as beans, sweet potatoes, and at the low end of the spectrum, spinach and rhubarb, exhibit much lower bioavailability for their calcium. Since the range of intrinsic bioavailability values spans something close to a full order of magnitude, it is clear that the available data in food tables do not give a clear picture of the *effective* calcium delivery for each. In the section that follows, I shall show data reflecting both the calcium content of various foods, and their bioavailability.

Food Sources and Bioavailability

For adults, dairy products supply 72% of the calcium in the U.S. diet, grain products about 11%, and vegetables and fruits about 6% (51). It is difficult for most individuals to ingest enough calcium from foods available in a cereal-based economy without including liberal amounts of dairy products. Ethnic and cultural practices, such as processing corn tortillas with lime, to some extent overcome the inherent limitation of cereal-based foods. Food manufacturers have recently developed a wider variety of calcium-fortified products that explicitly compensate for their low calcium content. Additionally, many individuals have turned to dietary supplements to meet their calcium needs. However, it is prudent to remember that calcium is not the only nutrient important to health that is supplied by dairy products. Users of milk in the U.S., compared to nonusers, get 35% more vitamin A, 38% more folate, 56% more riboflavin, 22% more magnesium, and 24% more potassium, in addition to 80% more calcium (52).

Aside from gross calcium content, potential calcium sources should be evaluated for bioavailability. Fractional calcium absorption from various dairy products is similar for comparable loads. The calcium from most supplements is absorbed approximately as well as that from milk, since solubility of the salts at neutral pH has little impact on calcium absorption (53). Absorption of one very soluble, complex salt, calcium-citrate-malate (CCM), is slightly to moderately better than that of most other salts (54).

Several plant constituents form indigestible salts with calcium, thereby decreasing absorption of calcium. The most potent inhibitor of calcium absorption is oxalic acid, found in high concentration in spinach and rhubarb and to a lesser extent in sweet potatoes and beans (55). Calcium absorption from spinach is only 4–5%, compared with 27–33% from milk ingested at a similar load (56).

Phytic acid, the storage form of phosphorus in seeds, is a modest inhibitor of calcium absorption (57). Fermentation, as occurs during bread making, reduces phytic acid interference because of the phytase present in live yeast (58). Since the early balance studies of McCance and Widdowson, who reported negative calcium balance while consuming whole wheat products (59), it has been assumed that fiber negatively affects calcium balance through either physical entrapment or cationic binding with uronic acid residues (60). However, it is more likely that the phytic acid associated with fiber-rich foods is the component that affected balance, since most purified fibers do not affect calcium absorption appreciably (61). Only concentrated sources of phytate such as wheat bran (58) or dried beans (62) substantially reduce calcium absorption. For other plants rich in calcium (primarily the *Brassica* genus, which includes broccoli, kale, bok choy, cabbage, and mustard and turnip greens), calcium bioavailability is as good as or better than that from milk (63), despite their rich fiber content (*see* Fig. 5).

Figure 7 summarizes these concepts graphically, plotting the amount of calcium absorbed from a typical serving of various natural foods. Absorbed calcium in this case

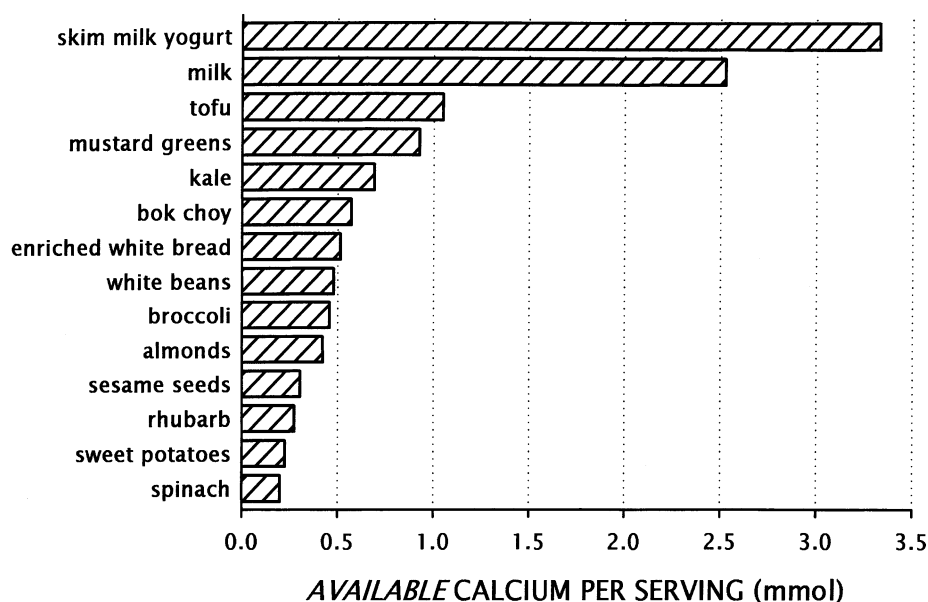


Fig. 7. Available calcium per serving for several natural foods. “Available” for this purpose means the product of calcium content per serving and measured bioavailability. (Copyright Robert P. Heaney, 1998. Used with permission.)

is the product of calcium content and measured bioavailability. The figure shows clearly why dairy sources are so important. Foods such as the *Brassica sp.* vegetables exhibit excellent calcium bioavailability (e.g., Fig. 5), but, as Fig. 7 demonstrates, depending upon them (and other generally available vegetable sources) to meet one’s intake needs would generally not be feasible.

Nutrient–Nutrient Interactions

Several nutrients and food constituents affect aspects of calcium homeostasis by means other than through the straightforward effect on digestibility and absorbability just described. Several dietary components influence urinary calcium excretion. One of the more important, mentioned briefly at the outset, is dietary sodium (6,7,64). Sodium and calcium share some of the same transport systems in the proximal tubule, so that each 100 mmol (2.3 g) increment of sodium excreted by the kidney pulls out approx 0.5–1.5 mmol (20–60 mg) of accompanying calcium. Because urinary calcium losses account for 50% of the variability in calcium retention, dietary sodium has a disproportionately large potential influence on bone loss. In adult women, each extra gram of sodium per day is projected to produce an additional rate of bone loss of 1%/yr if the calcium lost in the urine comes from the skeleton (65). A longitudinal study of postmenopausal women showed a negative correlation between urinary sodium excretion and bone density of the hip (66). The authors concluded, from the range of values available to them, that bone loss could have been prevented either by a daily increase in dietary calcium of 891 mg (~22 mmol) or by halving the daily sodium intake. As experienced clinicians will recognize, the latter option is not practicable.

Another dietary component that influences urinary calcium excretion is protein. Each gram of protein metabolized increases urinary calcium levels by about 0.025 mmol (1 mg); thus, doubling the amount of purified dietary proteins or amino acids in the diet increases

urinary calcium by about 50% (4). The acid load of the sulfate produced in the metabolism of sulfur-containing amino acids is believed to be mainly responsible for this effect. At the other extreme, inadequate protein intakes compromise bone health and may contribute to osteoporosis in the elderly. As discussed earlier (see Quantitative Operation of the System), high protein intakes are potentially harmful only in the face of low calcium intakes.

Although widely varying ratios of dietary phosphorus and calcium have not been associated with changes in adult calcium balance (67) (presumably because of the offset of increased endogenous secretion of calcium by decreased urinary calcium), some investigators have been concerned about the popular trend toward phosphate consumption in soft drinks. Acutely, phosphate loads cause increased circulating PTH secretion. Elevated PTH levels, if sustained, could lead to bone resorption. However, when bone resorption is measured, no such effect is found, and the Institute of Medicine found no cause for concern in current levels of phosphorus intake, noting, in fact, that human phosphorus intakes, when adjusted for energy, are at the extreme low end of the range of intakes for primates and laboratory animals (25).

Although caffeine in large amounts acutely increases urinary calcium (68), 24-h urinary calcium was not altered in a double-blind, placebo-controlled trial (69). Daily consumption of caffeine equivalent to 2–3 cups of coffee was associated with accelerated bone loss from the spine and total body in postmenopausal women who consumed less than 744 mg calcium per day (70). The relationship between caffeine intake and bone loss in this observational study may be due to a small decrease in calcium absorption (71) or to a confounding factor such as a probable inverse association between milk intake and caffeine intake.

Fat intake has a negative impact on calcium balance only during steatorrhea. In this condition, calcium forms insoluble soaps with fatty acids in the gut.

Increased use of calcium supplements and fortified foods has raised concern about high calcium intakes' producing relative deficiencies of several minerals. High calcium intakes have produced relative magnesium deficiencies in rats (72); however, calcium intake does not affect magnesium retention in humans (73). Similarly, except for a single report in postmenopausal women (74), decreased zinc retention has not been associated with high calcium intakes (75). The nature of this interaction is complicated and requires further study. Iron absorption from non-heme sources is decreased by half from radio-labeled test meals in the presence of calcium intakes up to 300 mg (7.5 mmol) Ca per day, above which there is no further reduction. However, up to 12 wk of calcium supplementation does not change iron status (76), and adolescent girls ingesting high calcium diets are able to increase total body iron mass as well as girls with low calcium intakes (77). This is probably because of compensating upregulation of iron absorption. Single-meal iron absorption studies inevitably exaggerate inhibitory effects that do not appear in the context of the whole diet and of physiological adaptation.

Fortified Foods

The notion of fortifying commonly used foods is an old one. The iodination of table salt is an example, and is clearly responsible for virtual elimination of iodine-deficiency goiter in countries that have adopted this practice. As the human population has moved into regions of the world that cannot provide the nutrients to which their physiologies have been adapted over the course of hominid evolution, they both need to discover what the missing nutrients may be and then find ways to supply them at a population level. Fortification has the advantage that anyone using the fortified foods gets the benefits,

without having to make a voluntary decision and without needing to adhere to that decision over the long-term. The nutrient concerned simply becomes a part of the food supply, just as it would have been under primitive conditions.

As noted earlier, the portions of plants that are lowest in calcium are the seeds, and, of course, the agricultural revolution has been based upon seed crops, principally cereal grains and legumes. Roughly 60% of the total energy intake of the world's population today comes from these seed food sources, whereas only a tiny fraction of the energy intake of the evolving hominid would have been from such foods. It makes sense, therefore, to enrich the calcium content of the seed foods, and such is beginning to happen in several countries around the world. Others reserve fortification as an instrument of national policy and do not permit voluntary fortification by manufacturers of the foods they produce. Admittedly, voluntary fortification is not always optimal as it often leads to high levels of enrichment of certain foods, rather than a general improvement of most of the food items in the diet. This is a rapidly evolving field, driven less by nutritional science than by market forces, and it is difficult to predict exactly what the aggregate impact of present practices will be.

Calcium is now added as various salts (principally calcium carbonate, calcium phosphate, and calcium sulfate) to a variety of foods, including ready-to-eat cereals, bread, cereal bars, energy drinks, and fruit juices. Not always has the bioavailability of the fortified product been tested. This is not just a theoretical concern. Experience has shown that interactions between the fortificant and other food constituents may affect bioavailability. So, as with natural foods, knowledge of the total calcium content of a fortified food may not be enough. For the next few years, at least, it will become increasingly difficult to assess a person's effective calcium intake, since it will be necessary to ascertain whether or not foods consumed were of a calcium-fortified variety, the level of fortification, and the bioavailability of the aggregate calcium content of the food.

In the best of all possible worlds, the fortification of foods would raise the calcium density of the total diet (i.e., mmol calcium per MJ of energy) to levels approximating that of the primitive diet, i.e., ~4.8 mmol/MJ. At such a time it would no longer be necessary to worry about or assess calcium intake, just as we no longer attempt to assess iodine intake.

Supplements

Calcium supplements will often be necessary in order to achieve desired total calcium intakes, although, with the growing availability of fortified foods, the need for supplements may well decline. Calcium supplements come in a large variety of forms involving different anions and different dosage units. Calcium carbonate and calcium citrate are probably the predominant forms, with the carbonate accounting for the lion's share of the total market. Calcium carbonate has been shown to exhibit good bioavailability (*see*, for example, Fig. 5). It is economical, it is well tolerated, and it exhibits close to the highest calcium density of any of the products available on the market. Most of the calcium supplements on the market today exhibit nearly equivalent bioavailability with some, such as calcium citrate malate or the calcium chelates exhibiting somewhat better fractional absorption. But generally the differences between the products are so minor as to be negligible, or to be easily offset by taking a single extra pill of a less expensive, if less well absorbed, product each week.

However, not all products are equally well formulated, in a pharmaceutical sense. While the pharmaceuticals of tablet disintegration and dissolution are well understood, the

supplement market, in the United States at least, is not held to those standards, and there was a period of time in the 1980s and 1990s during which varieties of calcium supplement tablets were widely distributed which simply did not disintegrate in the gastrointestinal tract, and hence enriched no one except their manufacturer. The United States Pharmacopoeia (USP) has established disintegration standards for calcium supplements, but adherence to these standards is voluntary, and regulation by the FDA in the United States is minimal. Hence the best course of action in the foreseeable future is to use a brand name supplement that adheres to USP standards or has been the subject of suitable bioavailability testing (or both).

Dose Timing

If the calcium is in the food, either naturally or through fortification, then timing is not a relevant issue, since the calcium will come into the body with the other nutrients of the food being consumed. However, if resort is had to calcium supplements, then timing can make a difference. Generally the safest course is to take calcium with meals. This replicates the primitive food pattern, and it tends to spread the intake out over the day, so as to optimize absorption (*see*, for example, Fig. 6). There are obvious problems involved in remembering to take multiple doses of medication, as well as adherence problems with pill-taking generally. (That is one of the reasons why a food fortification policy is likely to produce better population penetration than a strategy based on calcium supplements.)

An argument has been made for taking calcium supplements at bedtime, inasmuch as much of the PTH-mediated bone resorption occurs in the early hours of the morning, and a large calcium dose at bedtime has been shown to suppress PTH secretion. Whether, in the last analysis, this makes any difference to bone mass has not been established. At least one study suggests that this stratagem has little effect (78).

Drug Interference

Calcium is a nutrient and, as such, exhibits little or no interactions with most medications, as is true for most other nutrients as well. Calcium does interfere with iron absorption in single-meal tests, but, as noted above, does not impede the accumulation of total body iron stores in adolescent girls. Similarly, concern about negative interactions between calcium and zinc and magnesium has proved to be unfounded (73,75). This should not be surprising since, once again, calcium is a nutrient that was present in high concentration in the primitive diet. If its presence there had interfered substantially with other nutrients essential for the health of the human organism, we would never have survived as a species.

Calcium does not interfere with the action of calcium channel blockers, except insofar as their effect on the body may be indirectly influenced by the circulating level of PTH [and, correspondingly, $1,25(\text{OH})_2\text{D}$]. Calcium, on the other hand, may interfere with the absorption of the tetracycline antibiotics, since the tetracyclines strongly adsorb to calcium crystals, which may to some extent be present in the intestinal lumen when the diet is high in calcium. Large doses of calcium have also been reported to interfere with absorption of thyroxine (79), although the effect is small. Nevertheless individuals being treated for hypothyroidism should possibly separate their intakes of thyroxine and calcium.

Calcium as Co-Therapy

Till now the discussion has focused more or less exclusively on calcium as a nutrient. There is a sense, also, in which it can be considered co-therapy particularly in the

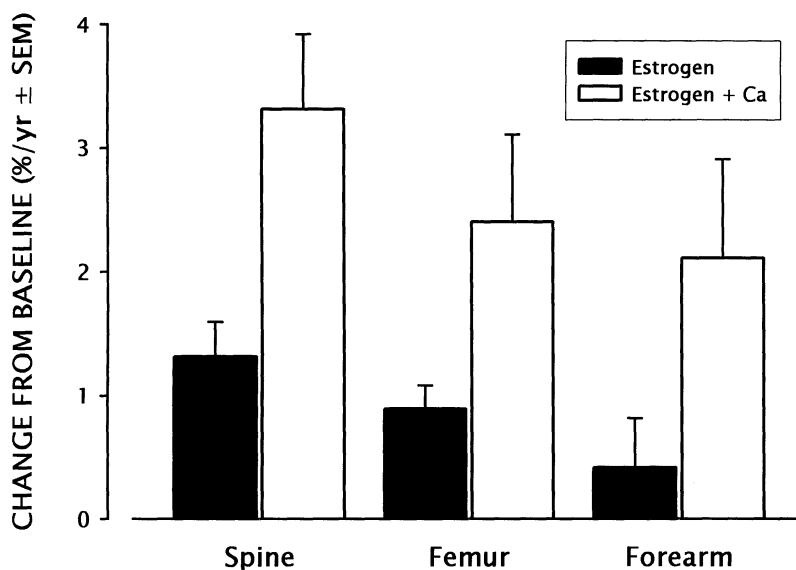


Fig. 8. Change in bone mineral density at three skeletal sites with estrogen replacement in postmenopausal women, both with and without supplemental calcium. (Redrawn from the data of ref. 37; Copyright Robert P Heaney, 2000. Used with permission.)

treatment of disorders such as osteoporosis. Here the knee jerk reflex has commonly been to think about treatment in terms of *pharmacotherapy*, ignoring the fact that bone is built of mineral, not of drugs or hormones, and that the efficacy of drug or hormone regimens may well depend to a substantial extent on providing adequate raw materials to build or maintain bone in the presence of bone-active pharmacologic agents. All of the modern bone active agents (bisphosphonates, SERMs, and PTH [80–84]) have been tested only in the effective presence of supplemental calcium, and it could be a mistake to conclude that they would remain effective if they were given without additional calcium. The additive effect of calcium is seen most clearly, perhaps, with respect to HRT, where there is extensive experience both with and without supplemental calcium. Figure 8 reproduces the data of a meta-analysis on this topic (37) showing the strongly additive effect of the addition of calcium to various HRT regimens.

While 32–46 mmol calcium per day may be sufficient for skeletal maintenance in an aging population, it will probably not be sufficient to produce optimal effects of the bone active agents in individuals with osteoporosis (85). Exact requirements for maximal effect are not known. But one anabolic agent, fluoride, produces an extraordinary degree of bone hunger, with a consequent requirement for calcium above 60 mmol/d (86).

Toxicity

Too much of any nutrient can produce signs of intoxication, and calcium is no exception. However, calcium intoxication, expressed principally as the milk alkali syndrome, is extremely rare, and has never been reported for food calcium sources. Pastoralist peoples of various ethnic backgrounds regularly consume 150–180 mmol calcium per day, life-long, without hint of adverse effects. All reports of this complication relate to supplement administration, mostly calcium carbonate.

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Clinical Aspects of the Use of Vitamin D and Its Metabolites in Osteoporosis

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INTRODUCTION

The vitamin D compounds regulate intestinal calcium absorption and thus contribute to the maintenance of serum calcium concentrations. They also have direct effects on bone, where their principal action is a stimulation of bone resorption. In addition, they impact on osteoblast activity, the kidney and a variety of other tissues. The clinical syndrome which develops in the presence of severe vitamin D deficiency is osteomalacia, presenting in children as rickets. Osteomalacia is characterized by the presence of unmineralized bone matrix. This occurs in vitamin D deficiency because the low concentrations of both calcium and phosphate in the extracellular fluid are inadequate to result in the normal formation of hydroxyapatite crystals. Osteomalacia caused by vitamin D deficiency is also characterized by marked hyperparathyroidism, as the body's homeostatic mechanisms struggle to maintain a normal serum calcium concentration in the face of its deficient intestinal absorption. Hyperparathyroidism results in the mobilization of calcium from bone.

Clinically apparent osteomalacia in adults is now regarded as a rarity. However, it is increasingly apparent that less marked degrees of vitamin D deficiency are common in those who do not venture outdoors regularly, particularly the frail elderly. These individuals also manifest secondary hyperparathyroidism and the associated acceleration of bone loss, though they often will not have evidence of unmineralized osteoid on bone biopsy. The hyperparathyroidism, however, contributes to the development of osteoporosis, so treatment of vitamin D deficiency becomes an important part of managing osteoporosis in many patients.

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The prevalence of vitamin D deficiency depends on sunlight intensity, sunlight exposure, and, to some extent, on dietary intake (1,2), and appears to be more common in Europe than in North America. It is particularly common in those living in institutions and in those requiring acute admission to hospital.

While the importance of vitamin D deficiency in accelerating postmenopausal bone loss is widely accepted, it is uncertain whether other changes in vitamin D metabolism contribute. In advanced old age, there may be a decline in serum concentrations of 1,25-dihydroxyvitamin D as a result of declining renal function, and this is likely to contribute to the rise in parathyroid hormone with age. How important this is to the development of postmenopausal osteoporosis is unclear and the variable results in trials of treatment using 1 α -hydroxylated vitamin D metabolites, suggests that this is a relatively small player in most individuals.

The above discussion makes clear the important distinction between supply of the parent compound (i.e., calciferol), and that of its numerous metabolites. There is good evidence for the biological activity of both 25-hydroxyvitamin D [25(OH)D] and 1,25-dihydroxyvitamin D, though the latter is by far the more potent. However, there have been suggestions that other metabolites such as 24,25-dihydroxyvitamin D may also have a role in skeletal metabolism. When calciferol is provided to a vitamin D deficient individual, all of these metabolites are replaced, and the body's homeostatic mechanisms determine the balance between them. In contrast, when a single active metabolite is administered (e.g., alfacalcidol or calcitriol) levels of other metabolites [e.g., 25(OH)D] may actually be reduced by this intervention, producing an unphysiological balance and a significant risk of hypercalciuria and hypercalcemia. Therefore, when reviewing the evidence from clinical trials, it is very important to draw a distinction between treatment of vitamin D deficiency with calciferol on the one hand, and use of its 1 α -hydroxylated metabolites on the other.

TREATMENT OF VITAMIN D DEFICIENCY

This is almost always undertaken with one of the calciferols, either ergocalciferol (vitamin D₂) or cholecalciferol (vitamin D₃), which are of plant and animal origin, respectively. They have generally been regarded as being equally potent, though several recent studies have suggested that compounds from the vitamin D₃ series have greater biological activity. In a few studies, 25(OH)D has been used. This would be theoretically attractive in situations where 25-hydroxylation in the liver was likely to be deficient. However, even in advanced liver disease there is usually sufficient capacity to provide adequate hydroxylation of vitamin D so calciferol is all that is necessary.

Recently, there have been attempts to determine the optimal serum 25(OH)D concentration, so that an appropriate threshold for intervention could be identified. A number of cross-sectional studies have addressed this by assessing the relationship between serum 25(OH)D and parathyroid hormone, using elevation of parathyroid hormone concentrations as a marker of vitamin D deficiency. Such investigations produce wide confidence intervals and some have suggested that the optimal serum 25(OH)D concentration may be as high as 100 nmol/L (40 μ g/L) (3). By this definition, the majority of postmenopausal women in most European populations would be defined as being deficient. Malabanan et al. (4) have addressed this issue by assessing whether the administration of vitamin D resulted in suppression of parathyroid hormone in individuals with

a variety of baseline serum 25(OH)D concentrations. They found that vitamin D supplementation only caused suppression of parathyroid hormone levels when baseline serum 25(OH)D was less than 50 nmol/L (20 µg/L). This level is still somewhat higher than the lower end of the reference ranges used in most laboratories (usually between 25–35 nmol/L [10–15 µg/L]), but does provide a more practical therapeutic target.

The most physiological way of replacing vitamin D is by exposing individuals to sunlight. Reid et al. (5) have demonstrated in a randomized controlled trial that spending 30 min/d outdoors results in increases in serum 25(OH)D levels from 24 to 31 µg/L. These changes had not plateaued at the end of the 1-mo study.

The oral or parenteral administration of calciferol is also effective. Daily doses between 400–1000 international units (IU) per day are typically used and virtually never cause vitamin D intoxication. (For calciferol, 1 µg is 40 IU). Indeed doses of up to several thousand units per day can be administered long-term without difficulties in most cases (6). Other regimens used include 50,000 IU of calciferol once a month by mouth, 100,000 IU every 3 mo by mouth (7), and 150,000 IU by annual intramuscular injection (8). Adams et al. (9) have recently described the use of a single oral dose of up to 500,000 IU of calciferol for treatment of established deficiency. The efficacy of intermittent regimens is attributable to vitamin D being a fat soluble vitamin which is stored in adipose tissue.

The effects of vitamin D supplementation on bone density have been studied in a number of contexts. In early postmenopausal women who are not deficient, supplementation has little if any effect on bone density (10). This was confirmed in a recent study in Finland, though there was a downward trend in numbers of fractures at 5 yr in those receiving 300 IU/d of calciferol alone, compared with placebo (11). However, these women would be considered to be vitamin D deficient by most standards, since their baseline 25(OH)D concentrations were 24–30 nmol/L (12).

In older women with sub-optimal serum 25(OH)D concentrations, as defined above, beneficial effects on bone mass of 1–2% are demonstrable (13,14). One study of patients with serum 25(OH)D < 14 µg/L demonstrated increases of 4% in spine and hip bone density with calciferol replacement (9).

There have now been two large studies of the effects of monotherapy with calciferol on fractures. Heikinheimo et al. (15) studied almost 800 elderly men and women, randomized to an annual injection of 150,000 IU vitamin D₂ or to control. Mean baseline levels of 25(OH)D were 31 nmol/L in the subjects living independently (two-thirds of the cohort) and 14 nmol/L in those in a municipal home. The respective groups increased to 49 nmol/L and 45 nmol/L with treatment. After a mean follow-up of 3 yr, symptomatic fractures were reduced by 25% in the vitamin D-treated subjects ($P = 0.03$). In contrast, Lips et al. (16) showed no difference in fracture incidence in 2578 independently-living men and women over the age of 70 yr randomized to receive calciferol 400 IU/d or placebo over a period of up to 3.5 yr. Mean serum 25(OH)D concentration in the third year of the study was 23 nmol/L in the placebo group and 60 nmol/L in the vitamin D group.

There have also been two large studies of combined treatment with calcium and calciferol in the elderly. In the 3-yr study of Chapuy et al. (17,18), more than 3000 women aged 69–106 yr living in institutions were randomly allocated to take placebo or 1.2 g of elemental calcium plus 800 IU of vitamin D₃ daily. Baseline serum 25(OH)D concentrations were 33–40 nmol/L and rose to 100–105 nmol/L in those receiving active therapy. At 18 mo, there were 32% fewer non-vertebral fractures in those receiving active treatment ($P = 0.02$) and 43% fewer hip fractures ($P = 0.04$). At the end of 3 yr of

treatment, the probabilities of nonvertebral fractures and hip fractures were reduced by 24 and 29%, respectively ($P < 0.001$), in those receiving active therapy. Proximal femoral bone mineral density (BMD) increased 2.7% in those receiving active treatment, and declined 4.6% in the placebo group ($p < 0.001$). Dawson-Hughes et al. (19) have reported similar findings. They randomized 389 men and women aged over 65 yr to treatment with either 500 mg of calcium plus 700 IU of calciferol per day or placebo. At the end of 3 yr, there had been new nonvertebral fractures in 26 subjects in the placebo group and in 11 in the calcium-vitamin D group ($P = 0.02$). In neither of these studies is it clear whether the calciferol, the calcium, or their combination were the key to success.

The data from these studies indicates that vitamin D deficiency and secondary hyperparathyroidism are common in frail elderly subjects, and suggest that these changes contribute to the progressive reduction in bone density which occurs in this age group. These biochemical abnormalities are reversible with physiological doses of vitamin D and calcium, and this results in beneficial effects on bone density and, more importantly, on fracture rates. Not only does this lead to a substantial reduction in morbidity, but is likely to be associated with a significant prolongation of life, since there was a 24% mortality amongst patients developing hip fractures in the Chapuy study.

CALCITRIOL IN POSTMENOPAUSAL OSTEOPOROSIS

Prevention

Christiansen's group have carried out two trials in cohorts of women diagnosed as having osteoporosis. The details of these are shown in the first two lines of Table 1. In the first of these, early postmenopausal women were randomized to placebo, calcitriol, hormone replacement therapy (HRT), or HRT plus calcitriol. Women taking HRT had increases in forearm bone mass of ~1% over one year whereas those on placebo or calcitriol lost 2%, the between-groups difference being significant. The same group carried out a similar study in a cohort of healthy 70-yr-old women, the starting dose of calcitriol in this study being 0.5 µg/d. Bone loss tended to be more marked in the calcitriol-treated group than in those taking placebo, and a loss of vertebral height was only observed in patients taking calcitriol. Recently, a study from Thailand compared the effects of calcitriol and HRT in early postmenopausal women (20). While calcitriol appeared to prevent the bone loss observed in the group given calcium alone, much more positive changes in bone mass were seen in those receiving estrogen. Taken together, these studies suggest that calcitriol has no place in the prevention of normal postmenopausal bone loss and, in European women, may even accelerate it.

Treatment

A larger number of studies have been carried out in women who already have osteoporosis, and these are also summarized in Table 1. Falch (21) failed to find any effects of calcitriol on forearm bone mineral content (BMC) and vertebral fractures over 3 yr in 76 women with a history of forearm fracture. The precision of the densitometer used was suboptimal (~3%), so a small effect may have been missed. Gallagher (22) has reported a two-center study assessing vertebral fracture incidence in 62 women over 1 yr. There was a substantial decrease in the fracture rate of the patients at one of the centers when a one-tailed test was used. By today's standards, this study is seriously under-powered.

A separate three-center US study has resulted in individual reports from each of the centers. Subjects with at least one vertebral fracture were randomized to calcitriol 0.5 µg/d

Table 1
Principal Randomized Controlled Trials of Calcitriol in Postmenopausal Osteoporosis

<i>Study</i>	<i>Subjects^a</i>	<i>Dose^b (µg/d)</i>	<i>n</i>	<i>Results</i>
Christiansen (45) ^d Jensen (46,67)	84, early postmenopausal 74, healthy 70-yr-olds	0.25 0.42	12 12	No effect of calcitriol on forearm BMC, beneficial effect of HRT Trend to ↓ forearm BMC with calcitriol. Beneficial effect of HRT Vertebral height decreased more in those taking calcitriol than placebo
Ongphiphadhanakul (20) Falch (21) Aloia (68)	146, early postmenopausal 76, forearm fracture 27, vertebral fracture	0.25 or 0.5 <0.5 0.8	24 36 24	Spine BMD: calcium ↓2.5%, HRT ↑5.4%, calcitriol 0.5 µg ↑1.1% Calcitriol not different from calciferol in changes in forearm BMC ↑ total body calcium, radius BMC and spine BMD, with calcitriol, by one-tailed tests
Gallagher (22) Ott (69) Fujita (70)	62, vertebral fracture 72, vertebral fracture 415, osteoporotic	>0.5 0.43 0.50	12 24 7	Reduced fracture rates at one of two centers using one-tailed tests ↓ distal radius BMC on calcitriol. No effects in total body or spine Calcitriol and alfacalcidol both maintain metacarpal BMD. No placebo group
Gallagher (71) Tilyard (23)	40, vertebral fracture 432, vertebral fracture	0.62 0.5	24 36	Calcitriol superior to placebo in total body BMD Stable fracture rate in calcitriol group, progressive rise in calcium group (significant)
Masud (51) Frediani (50)	47, vert frac or low BMD 102, low BMD	0.5 0.5	12 24	↑ spine & hip BMD: etidronate + calcitriol > etidronate Effects on total body BMD were: calcitriol+alendronate > alendronate > calcitriol > calcium

^aNumbers are those completing the study.
^bAverage dose of calcitriol in "active" group.
^cDuration in months.
^dReference number of study.

or placebo, with dose escalation until hypercalciuria or hypercalcemia occurred. The dose titration resulted in the patients of Ott and Chestnut receiving a mean dose of 0.43 µg/d, those of Gallagher 0.62 µg/d, and those of Aloia 0.8 µg/d. The Aloia patients showed between-groups differences in bone density of 2–4% at the various sites (significant using one-tailed tests) but had frequent hypercalciuria and hypercalcemia. The Gallagher group also found beneficial effects (2% between-groups difference in total body BMD at 2 yr) and averted major problems with hypercalcemia by restricting dietary calcium intake to 600 mg/d. Ott and Chestnut also had a good safety outcome by restricting calcium intake but bone density changes tended to be more positive in the placebo group than in those receiving calcitriol at the three skeletal sites assessed.

The largest trial of calcitriol in osteoporosis is that of Tilyard (23). Six hundred and twenty-two women with vertebral fractures were randomized to calcitriol 0.5 µg/d or calcium 1 g/d and followed for three years. The only end-point was vertebral fracture. There were significantly more fractures in the calcium group than in the calcitriol group in both years two and three—the fracture rate remained stable in the calcitriol group throughout the study but increased threefold in those taking calcium. This apparent deleterious effect of calcium supplementation may be contributed to by the large number of withdrawals in year one. Other issues with this study are that a number of the subjects were vitamin D deficient at entry, the lower fracture rates in calcitriol-treated subjects were only seen in those who had fewer than six fractures at baseline, and the study was not double-blind. Thus, it does not establish the antifracture efficacy of calcitriol.

At the time of writing, there are several new studies that have only been presented in abstract form. Fenton et al. (24) randomized postmenopausal women to receive HRT or calcitriol 0.5 µg/d. At 1 yr, the HRT group showed BMD increases of 6.2% at the spine and 3.6% at the hip, with no change in those receiving calcitriol. In a similar study, Gallagher (25) randomized 489 elderly women to HRT, calcitriol, neither, or both. At 3 yr, the increases in BMD were about twice as great in those taking HRT compared with those seen in calcitriol-treated patients, and combination therapy tended to produce the greatest increments in BMD. There was a trend for fracture rates to be lower in the calcitriol groups.

Finally, Fujita (26) has reported a large, double-blind trial comparing calcitriol 0.5 µg/d with alfacalcidol 1 µg/d. The two therapies appeared to have equivalent effects on metacarpal BMD over a period of 7 mo.

The above studies are generally reassuring regarding the safety of calcitriol use, particularly when doses do not exceed 0.5 µg/d. This dose, when used in the absence of calcium supplementation, causes only modest hypercalciuria. None of the more than 200 women treated over a 3-yr period by Tilyard et al. (23) developed renal colic, and in the 60 subjects who had renal ultrasonography after two years treatment, no evidence of calcium deposition was seen. In most studies, serum calcium levels have remained stable throughout the trial period, though there have been reports of hypercalcemia in routine clinical use of calcitriol (27). However, it is quite clear that a combination of calcitriol with calcium supplementation, or calcitriol doses >0.5 µg/d will result in significant hypercalciuria and hypercalcemia. Such patients requiring frequent monitoring.

ALFACALCIDOL IN POSTMENOPAUSAL OSTEOPOROSIS

Alfacalcidol (1α-hydroxycholecalciferol) is a synthetic vitamin D compound requiring hydroxylation at position 25 to form calcitriol, its active form. This conversion takes place rapidly in the liver, so it is effectively a pro-drug of calcitriol.

Table 2
Randomized Controlled Trials of Alfacalcidol in Postmenopausal Osteoporosis

Study	Subjects ^a	Dose ^b (µg/d)	t ^c	Results
Christiansen (10) ^d	126 early postmenopausal	0.25	24	No effect on forearm BMC
Itoi (28)	64 early postmenopausal	1	24	Comparable bone loss with calcium and 1α(OH)D (-12%), none with HRT
Gorai (29)	79 early postmenopausal	1	24	Spine BMD: CE +2%; 1α(OH)D+CE +3%; 1α(OH)D and control -3%
Sorensen (30)	26 with vertebral fractures	2	3	Forearm BMC ↑14%
Hoikka (31)	37 with hip fracture	1	6	No effect on radial BMD
Itami (33)	25 with osteoporosis	1	12	Significant effect on radius and spine
Shiraki (32)	78 with osteoporosis	0.5/1	6	Significant effect on radial BMD
Fujita (26)	299 with osteoporosis	0.75	7	Significant effect on metacarpals
Orimo (34)	86 with vertebral fracture	1	≈24	↓ fracture rate with 1α(OH)D and 1α(OH)D + Ca
Fujita (37)	406 with osteoporosis	1	11	↑ spine BMD and ↓ fractures with etidronate, no change with 1α(OH)D
Menczel (35)	46 with osteoporosis	0.5	36	Radial BMC ↑ 0.3%/y with 1α(OH)D, ↓ 2.6%/y with placebo
Orimo (38)	74 with osteopenia + fracture	1	12	Significant effect on spine BMD. ↓ vertebral fractures
Lyritis (36)	64 with vertebral fracture	1	12	Radial BMC: nandrolone ↑ 5%, 1α(OH)D ↓ 2.8%
Shiraki (72)	43 with osteoporosis	0.75	24	Spine BMD: 1α(OH)D ↑ 2.3%, placebo ↓ 0.3% - not significant
Chen (40)	45 with low BMD	0.75	12	Spine BMD: 1α(OH)D ↑ 2.1%, calcium ↓ 2.1% - significant
Shiraki (41)	163 with osteoporosis	1	12	Spine BMD: 1α(OH)D ↑ 1.4%, alendronate ↓ 6.2% - significant

^aTotal number completing study.

^bAverage dose of alfacalcidol in "active" group.

^cDuration in months.

^dReference number of study.

CE, conjugated estrogens.

Table 2 summarises the principal randomised controlled trials of alfacalcidol in the management of postmenopausal osteoporosis. Again, there have been a small number of trials in normal, early postmenopausal women, and a larger number in those with osteoporosis diagnosed in one way or another. Christiansen (10) included a low dose of alfacalcidol as one of the arms in a large double-blind study of osteoporosis prevention in early postmenopausal women. There was no evidence of any slowing of bone loss in the women receiving alfacalcidol, whereas HRT and thiazide diuretics showed significant beneficial effects on forearm BMC at the end of the 2-yr study period. There have been two recent studies of alfacalcidol in the prevention of early postmenopausal bone loss, each of which also included groups treated with estrogen (28,29). Neither demonstrated any advantage of alfacalcidol over calcium or control. In contrast, HRT prevented bone loss in both studies, though there was a nonsignificant trend for combination therapy to be superior, in the study of Gorai et al. Thus there is no evidence to support the use of alfacalcidol in the prevention of bone loss in non-osteoporotic women.

Of the studies in osteoporotic women, some lasted only a few months or had small numbers, and these have produced inconsistent results. Substantial increases in forearm BMC were observed in a group of Danish women (many of whom appear to have also had osteomalacia) (30), whereas Hoikka found no effect on the same end-point in women recovering from hip fracture (31). Shiraki et al. (32) showed that both alfacalcidol 0.5 µg/d and 1 µg/d were superior to placebo in their effects on radial BMC, though the sizes of the groups were quite different, suggesting that the assignment of subjects was non-random. A large multicenter Japanese study (26) randomized 300 osteoporotic patients to alfacalcidol 0.75 µg/d or placebo, over a 7-mo period. At 3 mo, differences in metacarpal thickness of >1%, and in metacarpal density of 5% had developed between the groups, and were maintained. An early study by Itami (33) also demonstrated large changes in forearm and spine density at 6 mo, though their finding of a 12% fall in densities at both sites in the placebo group implies major imprecision in the measurement of bone density in this study. Orimo (34) carried out a 2-yr fracture study in 86 women, using a factorial design to assess calcium supplementation and alfacalcidol. It is not clear that the trial was formally randomized and baseline fracture prevalences varied among the groups. The rates of new vertebral fractures were lower in the groups taking alfacalcidol.

Two medium-sized studies have assessed the effects of alfacalcidol on the forearm alone. Menczel (35) found that BMC remained stable in the alfacalcidol-treated subjects but declined at an average rate of 2.6%/yr in those receiving placebo (between-groups difference, $P < 0.05$). The control group was augmented by pooling it with that from another study—no data are presented to allow comparisons solely within the original randomized cohort. In contrast, Lyritis et al. (36) found ongoing bone loss in women taking alfacalcidol compared with a 5% gain in forearm BMC with nandrolone. There was no untreated comparator group.

A similar design was used in the largest study of alfacalcidol (37), with etidronate as the active comparator. Patients were randomized to either alfacalcidol or to one of two cyclical etidronate regimens (either 200 mg/d or 400 mg/d for the 2-wk treatment period). At 1 yr, lumbar spine bone density increased 2.4 or 3.4% for the respective etidronate regimens, but showed a non-significant decline (−0.5%) in those receiving alfacalcidol ($P < 0.001$ vs both etidronate groups). Fracture rates (per hundred patients) were: low-dose etidronate, 6.9; high-dose etidronate, 5.4; alfacalcidol, 15 ($P = 0.028$ vs high-dose etidronate group).

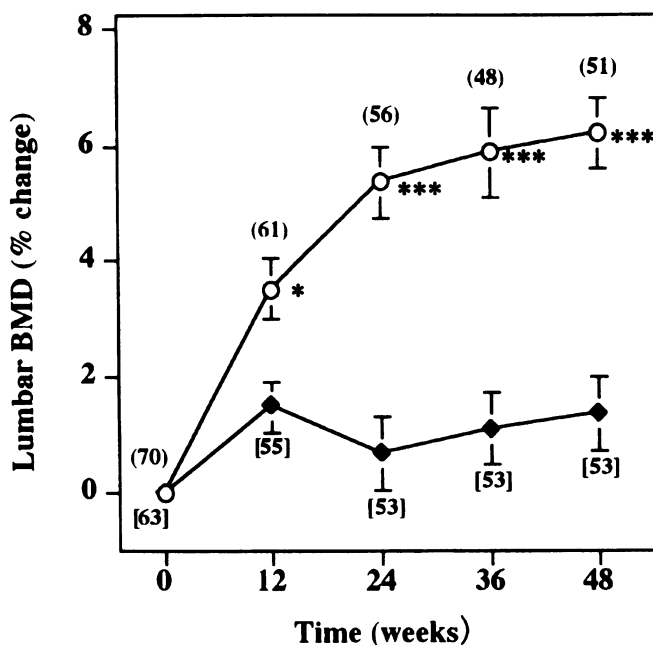


Fig. 1. Comparison of the effects of alendronate 5 mg/d (open circles) and alfacalcidol 1 µg/d (closed diamonds) in Japanese patients with osteoporosis. Data are mean \pm SE. The numbers in brackets represent the number of patients at that time-point. The significance of between-groups differences is shown: *, $P < 0.05$; ***, $P < 0.001$. Reprinted with permission from ref. 41.

There are now several medium-sized studies which have assessed alfacalcidol using modern techniques of axial bone densitometry. Orimo et al. (38) used dual-energy x-ray absorptiometry of the lumbar spine and femur in a study of 74 women (but as few as 34 were included in some analyses [e.g., femoral bone density] because of missing data). In the lumbar spine, the change in bone density was +0.65% in the alfacalcidol group and -1.14 % in the placebo-treated subjects ($P = 0.04$). There was no significant treatment effect in the proximal femur. Shiraki et al. (39) and Chen et al. (40) have also studied women with osteoporosis, and reported modest increases in spine BMD of about 2% at 1–2 yr. In the Shiraki study, the numbers of subjects with data for each parameter reported varied widely because not all subjects underwent all assessments and because almost half of the bone density measurements were judged to be technically unsatisfactory. The Chen study achieved a 90% retention of subjects, but was not blinded. Despite these differences, the results of these studies are very similar. Shiraki has recently reported another study (41) in which alfacalcidol and alendronate were compared over 48 wk. Lumbar spine BMD increased slightly in those receiving the vitamin D metabolite, but substantially more in those on alendronate (Fig. 1). No proximal femoral data were reported.

A further double-blind, randomized, placebo-controlled trial has not been included in Table 2 since it is not strictly in postmenopausal osteoporosis (42). This was carried out in 86 elderly patients (51 women) with Parkinson's disease who were randomized to alfacalcidol 1 µg/d or to placebo. The majority were vitamin D deficient at baseline. At 18 mo, density of the second metacarpal decreased 1.2% in the alfacalcidol group compared with a loss of 6.7% in the placebo group ($p < 0.0001$). Much of this response may represent the effect of treating vitamin D deficiency.

As with calcitriol, the principal safety issues with alfacalcidol are the risks of hypercalciuria and hypercalcemia. In Japanese patients, alfacalcidol 1 µg/d does not elevate serum calcium concentrations unless it is taken in combination with a calcium supplement (34,43). In other groups, there may be hypercalciuria with only 0.5 µg/d if calcium supplements are also used.

Taken together, these studies suggest a modest beneficial effect of alfacalcidol in postmenopausal osteoporosis. None of the studies establish its efficacy definitively, and those using active comparators suggest it is clearly inferior to either an anabolic steroid or a weak bisphosphonate. Virtually all the positive data comes from studies in Japanese subjects and may not be generalizable to European populations because of genetic and lifestyle (e.g., dietary calcium) differences.

COMBINATION REGIMENS

The combination of vitamin D and its metabolites with other therapies for osteoporosis has been common, though only recently has evidence to support the practice been forthcoming. When combined with fluoride, vitamin D prevents the development of secondary hyperparathyroidism and the resulting loss of cortical bone (44). Early studies of calcitriol with HRT suggested no benefit (45,46) from using both together, and similar findings came from the use of calcitriol with a bisphosphonate (47) or calcitonin (48). Recently, however, studies have shown benefit from the addition of calcitriol to either HRT (49) or a bisphosphonate. Frediani et al. (50) randomized women to take calcium, calcitriol 0.5 µg/d, alendronate (10 mg/d), or both (Table 1). At 2 yr, the approximate changes in total body BMD were -2, +2, +4, and +6, respectively, the combination therapy being significantly better than any of the other interventions. Masud et al. (51) compared cyclical etidronate with this regimen plus calcitriol (Table 1). Again, there was a benefit of more than 2% in the BMD changes at both the spine and hip. These findings suggest that vitamin D metabolites have small additional positive effects on bone density when their stimulation of bone resorption is blocked by the coadministration of an antiresorptive agent.

MALE OSTEOPOROSIS

Orwoll et al. (52) studied the effects of calciferol 1000 IU/d plus calcium 1000 mg/d in a placebo-controlled trial in normal men aged 30–87 yr. Seventy-seven men were studied over a 3-yr period. There was no difference in rates of change of either radial or vertebral BMD between the two groups. However, some of the more recent studies of vitamin D replacement have included men (15,19) and they suggest that the beneficial effects are uniform between the sexes.

Ebeling (53) recently presented a preliminary account of a randomized, double-blind, placebo-controlled trial of calcitriol 0.5 µg/d vs calcium 1 g/d in osteoporotic men with at least one baseline fracture. The calcium group showed transient positive changes in bone density at the hip and spine, though at 2 yr there were no differences between the groups. Over the 2 yr of the study, there were 15 vertebral and 6 nonvertebral fractures in the calcitriol group but only a single vertebral fracture in those taking calcium ($p = 0.03$). This suggests that calcitriol should not be used in idiopathic male osteoporosis.

GLUCOCORTICOID-INDUCED OSTEOPOROSIS

Trials in this area have recently been reviewed in detail (54). Two conflicting studies of calcium and vitamin D combinations have been reported. Buckley et al. studied patients with rheumatoid arthritis receiving low-dose prednisone who were randomized to receive placebo or calcium 500 mg/d plus vitamin D 500 IU/d (55). Those receiving calcium and vitamin D showed 5% more positive changes in bone density than those receiving placebo. The vitamin D status of the study subjects was not assessed. In contrast, Adachi et al. (56) failed to show any benefit on lumbar spine density from the use of calciferol 50,000 U/wk plus calcium 1000 mg/d in a randomised controlled trial over 3 yr.

Two small studies have demonstrated benefit from the use of 25-hydroxyvitamin D, but this agent is not widely available (57,58). Since much of a dose of calciferol is converted to 25(OH)D, it is not clear that the effects of these two agents would be expected to be different.

Calcitriol has been assessed in several randomized controlled trials. Dykman et al. (59) found no difference between calcitriol 0.4 µg/d and placebo in their effects on forearm bone density. Sambrook et al. (60) have reported a 1-yr study in which patients beginning glucocorticoid therapy were randomly assigned to receive calcium, calcium plus calcitriol (mean dose 0.6 µg/d) or these two agents combined with calcitonin. Bone losses from the lumbar spine were 4.3%, 1.3% and 0.2% in the respective groups. There was a similar, nonsignificant trend in distal radial bone loss but no evidence of reduced bone loss in the proximal femur (3% in all groups). A recent large trial over 3 yr in cardiac transplant patients showed no effect on lumbar spine density from calcitriol 0.25 µg/d (61), and similar findings have been reported after renal transplantation also (62). A trial comparing the use of calcitriol 0.5 µg/d with HRT in hypogonadal young women with systemic lupus erythematosus, showed progressive bone loss in those taking the vitamin analog in comparison with increases in density observed in those receiving hormones (between-groups difference at the spine of 3.7% at 2 yr) (63). There was also a significant difference between-groups at the distal radius.

Alfacalcidol has been studied by Braun et al. who demonstrated a beneficial effect at a dose of 2 µg/d on trabecular bone volume over a six month period. Following cardiac transplantation, alfacalcidol has been shown to slow but not completely prevent femoral neck and lumbar spine bone loss (64). A similar attenuation of lumbar spine bone loss has been reported in a predominantly non-transplant population with the use of alfacalcidol 1 µg/d, though femoral bone density was not measured in this study (65). In a population of patients with established steroid osteoporosis, Ringe has shown a beneficial effect at the lumbar spine (2.5% between-groups at 3 yr) of alfacalcidol 1 µg/d in comparison with calciferol plus calcium supplements (66). There was no significant effect in the proximal femur. Thus, there is some consistency in the results with this particular agent.

CONCLUSIONS

Among elderly subjects in many countries, there is consistent evidence of vitamin D deficiency and that it results in secondary hyperparathyroidism and accelerated bone loss. The treatment/prevention of this problem with physiological doses of calciferol

(possibly with calcium) has beneficial effects on bone mass and fracture incidence and should be vigorously promoted.

The use of vitamin D metabolite therapy, however, is much more controversial. There is no evidence to support its use in the prevention of bone loss in normal postmenopausal women. In osteoporotic women, trials have produced variable results, except in Japan where outcomes have been more consistently positive. In those studies in which beneficial effects on BMD have been found, these have generally been less than are seen with HRT or bisphosphonates. Vitamin D metabolites may have additive effects on BMD to those of other antiresorptive agents. There is no conclusive evidence for antifracture efficacy of these compounds. The remaining uncertainties regarding the active metabolites of vitamin D will require considerably more data for their resolution, but whether these studies will be completed or whether the vitamin D metabolites will be bypassed as more promising novel therapies are developed is unclear at the present time.

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The Basic Biology of Estrogen and Bone

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INTRODUCTION

Estrogen* influences most, if not all, aspects of bone physiology during postnatal life, including bone growth, architecture and turnover (Table 1). This review will focus on recent advances in understanding the physiological actions of estrogen on mammalian bone. The very interesting action of estrogen on bone and mineral metabolism in other classes of vertebrates has been reviewed elsewhere (1). The reader is also directed to more comprehensive reviews of the in vitro response of bone cells to estrogen (1,2).

*“Estrogen” refers to a family of molecules which are related to one another by structure or activity. This family includes the sex hormone 17 β -estradiol, metabolites of the hormone, synthetic hormone analogs including environmental estrogens and phytoestrogens. In this review, unless a specific compound is indicated, “estrogen” refers to the composite actions of this family.

Table 1
Effects of Estrogen on Rat Tibia

<i>Measurement</i>	<i>Juvenile</i>	<i>Adult (>8 mo old)</i>
Architecture		
Cortical bone		
Length	↓	NC
Cross-sectional area	↓	NC
Marrow area	↓	NC
Cortical thickness	NC	NC
Cancellous bone		
Metaphysis		
Bone volume	↑	NC
Trabecular number	↑	NC
Trabecular thickness	NC	NC
Trabecular separation	↓	NC
Growth plate		
Height	↓	—
Epiphysis		
Bone volume	NC	NC
Cells		
Cortical bone		
Periosteum		
Osteoblast number	↓	↓*
Osteoblast activity	↓	↓*
Endocortical		
Osteoblast number	NC	NC
Osteoblast activity	NC	NC
Osteoclast number	↓	?
Osteoclast activity	?	?
Cancellous bone		
Metaphysis		
Osteoblast number	↓	↓
Osteoblast activity	↓	↓
Osteoclast number	↓	↓
Osteoclast activity	?	?
Growth plate		
Chondrocyte proliferation	↓	—
Chondroclast number	↓	—
Chondroclast activity	?	?
Epiphysis		
Osteoblast number	↓	↓
Osteoblast activity	↓	↓
Osteoclast number	↓	↓
Osteoclast activity	?	?

↓, Decreased; ↑, increased; NC, no consistent change; —, not present in adults; * Rate of periosteal growth is greatly reduced compared to juvenile.

ESTROGEN AND PEAK BONE MASS

Endochondral Ossification

Bone length increases by endochondral ossification, the process by which cartilage cells in the growth plate proliferate, expand in volume and are replaced by bone following vascular invasion. The growth rate decreases after a pubertal growth spurt and terminates following epiphyseal closure.

Estrogen influences bone length and cancellous bone mass in many mammalian species by regulating the above-mentioned processes but the architectural and cellular changes have been described in greatest detail in rats. Estrogen antagonizes the proliferation of chondrocytes in the growth plate in rats, which reduces the rate of bone elongation (3). However, estrogen also decreases chondroclast number, which delays epiphyseal closure. Estrogen decreases chondroclast number, in part by antagonizing the fusion of the mononucleated circulating chondroclast precursor cells (4).

Surprisingly, the growth suppressing actions of estrogen can lead to an increase in cancellous bone density. The well documented inhibitory action of estrogen on chondroclast-mediated vascular invasion of the calcified zone of the growth plate of rats results in an increase in primary spongiosa which in turn results in a proportional increase in cancellous bone. Thus, estrogen can greatly increase cancellous bone volume in growing mammals by a nonanabolic mechanism. Indeed, this increase in bone volume is accompanied by a decrease in bone formation rate (4–6).

The inhibitory effects of estrogen on growth plate regeneration, resulting from chondrocyte proliferation, and growth plate destruction, due to vascular invasion of the growth plate, represent opposing actions on growth plate survival. Continuous exposure to estrogen results in a condition whereby vascular invasion exceeds growth plate regeneration. As a consequence, ovariectomy results in increased longitudinal bone growth and delayed epiphyseal closure whereas administration of estrogen to ovariectomized animals decreases bone growth and accelerates growth plate closure (3–6).

Intramembranous Bone

The volume of the medullary canal in rat tibiae is enlarged following ovariectomy due to a net increase in bone resorption (7,8). Osteoclast number is increased (8) and bone formation remains unchanged or increased (7–9). In contrast, there is an increase in bone formation at the periosteal surface (7) which is suppressed by 17 β -estradiol (7,10,11). As a result of the opposing changes in radial growth and endocortical modeling, the cortical bone volume changes little in adult ovariectomized rats. Cortical bone mass may increase in rapidly growing ovariectomized rats because the increase in periosteal bone formation exceeds the increase in endocortical resorption (10). The cellular mechanism for the differential response of the periosteal and endocortical bone surfaces of the midshaft appears to be related to the different functions and populations of cells that comprise the two bone envelopes (7,11).

Under normal circumstances, bone resorption at the periosteum of growing rats is limited to developing vascular spaces. Osteoclasts are uncommon at other locations on the periosteum and are not notably increased following ovariectomy (11).

One of the limitations of rat and mouse studies is that the skeleton of small rodents does not undergo cortical bone remodeling. Ovariectomy increases endocortical bone resorption surface and cortical porosity in dogs (12). The endocrinology of the dog limits this species usefulness as a model for postmenopausal bone changes. As a consequence,

studies in other large animals with an estrus cycle more similar to the human menstrual cycle (e.g., nonhuman primates) are needed to fully establish the effects of sex steroids on Haversian (cortical) bone remodeling.

The low level of periosteal resorption is contrasted with the endocortical surface, which undergoes aggressive bone modeling during growth to increase the volume of the marrow cavity. Treatment of ovariectomized rats with estrogens prevents the increase in medullary area by preventing the increase in osteoclast number (7,8).

The mechanisms for the growth inhibitory actions of estrogen on cortical bone are incompletely known. At the cellular level, estrogen reduced the number of preosteoblasts in S-phase of the cell cycle, suggesting that the hormone inhibits the production of osteoblasts (5,11). At the same time, estrogen decreases the steady-state mRNA levels for bone matrix proteins and IGF-I, reduces bone matrix synthesis, reduces osteoblast number and increases the population of bone lining cells (10,11). Estrogen inhibits bone growth by decreasing bone formation as well as bone resorption and these changes are associated with decreased expression of bone cell survival factors such as IGF-I.

The rat has been a valuable laboratory animal model but there are species as well as site specific differences which preclude generalizing the effects of estrogen on bone cells. For example, high dose 17 β -estradiol can result in an osteosclerotic response in ovariectomized as well as ovary intact mice whereas the hormone inhibits bone formation in the rat (5,13).

PREGNANCY AND LACTATION

A comprehensive discussion of changes in bone and mineral homeostasis that occur during pregnancy and lactation is beyond the scope of this review. The reader is directed to the excellent review by Kovacs and Konnenberg which focuses on the changes that occur during human reproduction (14).

Briefly, pregnancy and lactation result in profound changes in bone and mineral metabolism in a wide variety of animals, including humans, nonhuman primates, dogs and rats. Calcium absorption increases as do indices of bone turnover (15). Bone mass decreases, predominantly at cancellous bone sites, with the major bone loss in humans occurring in the vertebrae (16,17). In general, lactation is responsible for more bone loss than pregnancy. Bone mass and turnover return to normal values following a return to menses, suggesting that ovarian dysfunction in general and decreased circulating estrogen in particular is responsible for the elevated turnover and bone loss (18,19).

ESTROGEN AND REGULATION OF BONE REMODELING

Rate of Bone Remodeling

Ovariectomy results in severe cancellous osteopenia in long bones and vertebrae of rats (20) and vertebrae of monkeys (21,22). The response to ovariectomy in dogs has been less consistent, with no change (23) and bone loss (24) reported. The rate of bone loss from the rat vertebrae occurs more slowly than from long bones (20,25). There are also regional differences within bones. Cancellous osteopenia is prominent in the proximal tibial metaphysis and is almost absent in the proximal epiphysis. This differential rate of bone loss may be related to differences in prevailing levels of mechanical loading (26). Although the mechanism for the interaction between estrogen and loading is incompletely understood, there is strong evidence for an overlap in the transduction pathways for the two signals on bone (27).

Ovariectomy results in increases in osteoblast-lined perimeter, osteoclast-lined perimeter, and osteoclast size in long bones of rats (28,29). There are simultaneous increases in the mineral apposition and bone formation rates, suggesting that ovariectomy results in chronic high bone turnover. Cancellous bone turnover remains elevated in rats a year or more after ovariectomy (20,30). Bone formation is increased in ovariectomized monkeys (21), suggesting the bone loss in nonhuman primates is also associated with increase bone turnover.

Replacement of estrogen stabilizes cancellous bone volume in ovariectomized rats by reducing the rate of bone turnover, re-establishing a neutral or positive balance between bone formation and bone resorption, and by preventing the destruction of trabecular plates (25,31,32). The target cells for estrogen action may include osteocytes (33) as well as osteoblasts and osteoclasts. Estrogen antagonizes initiation of bone remodeling, in part, by decreasing the fusion of osteoclast precursors (4). The hormone may also reduce osteoclast number decreasing osteoclast lifespan (34).

The effects of estrogens on cancellous bone formation are controversial. There is general agreement that the inhibitory effects of the hormone on initiation of bone remodeling in rats leads to an overall coupled decrease in bone formation as well as improved bone remodeling balance. The latter change may be due to increased osteoblast lifespan (35). On the other hand, some investigators have reported that 17β -estradiol has separate direct stimulatory actions in rats to increase osteoblast number and bone formation (36,37). Most investigators, however, have reported that the hormone inhibits indices of bone formation, including osteoblast number and activity (25,38). Studies designed to investigate this controversy failed to reveal any evidence for an anabolic action of 17β -estradiol on bone formation in rats (5). Furthermore, recent time course studies have shown that the hormone results in rapid decreases in mRNA levels for bone matrix proteins and collagen synthesis (39). Finally, no investigator has been able to demonstrate restoration of bone volume with estrogen in adult rats with established bone loss (40).

There are species differences in the response of osteoblasts to 17β -estradiol. Whereas there is no compelling evidence that the hormone directly stimulates the rate of bone formation in either rats or humans, there is undeniable evidence that 17β -estradiol increases bone formation on endocortical surfaces as well as induces de novo cancellous bone formation in the marrow cavity of mice (13,41). However, these actions in mice do not occur at physiological levels of 17β -estradiol and may occur secondarily to cytotoxic effects of the hormone on bone marrow (42).

Remodeling Balance

There is overwhelming evidence that 17β -estradiol reduces the overall rate of bone remodeling but the effects of the hormone on remodeling balance are less certain. Estrogen replacement was reported to increase trabecular mean wall thickness in postmenopausal women suggesting that in the presence of the hormone the volume of bone removed during the resorptive phase of the remodeling cycle is exceeded by the volume of new bone reproduced during the formation phase, leading to a positive remodeling balance (43). In rats, ovariectomy results in dramatic decreases in trabecular number with little or no decrease in trabecular thickness (44). Ovariectomy was reported not to alter mean wall thickness in rats (45), but additional studies in older rats should be performed to verify these results. Taken as a whole, these observations suggest that the principal effect of estrogen on bone turnover in adults is to regulate the remodeling rate but that under some circumstances the remodeling balance is improved in the hormone's presence.

ESTROGEN TARGET CELLS IN BONE

The available evidence suggests that only a subset of osteogenic cells in bone tissue expresses the two known isoforms of the estrogen receptor, ER- α and ER- β . Although the distribution of ER- α and ER- β in bone is incompletely known, the pattern of expression of the two isoforms appears to overlap. Both ER- α and ER- β have been localized in hypertrophic chondrocytes in growth plate (46,47) and ER- α is additionally present in chondrocytes of the proliferating zone (46,48). In bone, both receptor isoforms are usually localized in osteoblasts and lining cells of cancellous bone, with lower levels in osteocytes and low to undetectable levels in osteoclasts. However, other patterns of distribution of ER- α have been reported. For example, immunofluorescence detection of ER- α in human female and pig bone revealed strongest reactivity in a subpopulation of the osteocytes with indistinct staining of osteoblasts and lining cells (49). Using a more sensitive *in situ* reverse transcriptase PCR technique to identify ER- α mRNA in actively remodeling human fracture callus, Hoyland et al. (50) showed that osteoblasts expressed the highest levels of ER- α , while osteocytes and osteoclasts expressed lower levels. These results were confirmed in another study, in which the ER- α mRNA and protein were similarly localized to osteoblasts and lining cells of human and rabbit cancellous bone, with no clear expression in osteocytes or osteoclasts (48).

The *in situ* RT-PCR technique has demonstrated a loss of ER- α mRNA expression from osteoblasts and osteocytes in males with idiopathic osteoporosis, which suggests estrogen resistance contributes to the etiology of the disease (51). In an immunolocalization study of mouse and human cancellous bone and fracture callus, ER- β was identified in osteoblasts, osteocytes and osteoclasts, with the latter cells exhibiting cytoplasmic rather than nuclear immunoreactivity (52). Similar results were obtained for ER- β mRNA in neonatal rat bone in which the transcripts were predominantly expressed by osteoblasts covering metaphyseal cancellous bone surfaces with lower signals in osteocytes and bone marrow (53). In rats, the relative levels of ER- α and ER- β mRNA have been reported to be lower in cortical compared to cancellous bone, with ER- α being the dominant species in both locations (39,54,55). The significance of this observation is unclear because cortical bone is highly responsive to estrogen and the most striking skeletal phenotype in ER- β knockout mice is an increase in cortical bone mineral content in postpubertal females. Although these gene expression studies suggest that the concentrations of ER- α are higher than ER- β in bone tissue as well as in osteoblasts at different stages of differentiation it is important to remember that the number of functional receptors have not been measured in skeletal tissues for either receptor isoform. It is also unclear what concentration of each receptor isoform is required for responsiveness in osteoblast cells and whether one isoform modulates the activity of the other when both are expressed, as has been reported in other cell types.

ANIMAL MODELS FOR POSTMENOPAUSAL OSTEOPOROSIS

Ovariectomized Rodents

The observation that acute ovarian hormone deficiency leads to elevated cancellous bone turnover dramatically increased interest in the rat as a model for postmenopausal osteoporosis (56). Subsequent studies showed that ovariectomy results in cancellous and cortical bone loss (7,8,20,25,28). The widespread adoption of this model by the pharma-

cological industry has played an important role in the successful development of novel therapies to prevent and/or treat osteoporosis, including antiresorbing agents such as bisphosphonates, selective estrogen receptor modulators (SERMs) and anabolic agents such as parathyroid hormone.

The cancellous osteopenia which occurs following ovariectomy in rapidly growing rats is primarily due to altered bone growth and thus is mediated by a mechanism which differs significantly from postmenopausal bone loss (4,5). In contrast, ovariectomy of skeletally mature rats is similar to menopause in that the surgery leads to cancellous and endocortical bone loss which is due primarily to abnormal bone remodeling. Similar bone loss can be induced in female rats by LHRH agonists and estrogen receptor antagonists (57,58). These alternatives to ovariectomy are reversible and have proven to be very useful for investigation of the bone loss associated with endocrine treatment of endometriosis. The predictive value of the ovariectomized rat is illustrated by the initial recognition of tissue selective actions of tamoxifen and other estrogen receptor ligands in that model (8). Subsequent confirmation in humans and development of selective estrogen receptor modulators (SERMs) for prevention of osteoporosis were a direct result of the initial animal observations (59).

The mouse is the premier laboratory animal model for studying the genetic contribution to peak bone mass and age-related bone loss. There are numerous well characterized mouse strains with differences in bone mass and response to comorbidity factors. Additionally, transgenic technology allows the purposeful manipulation of specific gene expression. There is a long and growing list of transgenic mice with perturbed bone metabolism (60). Improvements in the ability to dynamically regulate genes in specific cell types will further increase the power of the mouse model. These genetic manipulations are not without pitfalls when applied to osteoporosis. Demonstration that a gene is associated with bone mass in the mouse does not necessarily mean that it has any role in the pathogenesis of osteoporosis. It will be essential to demonstrate that there is a cause and effect relationship in humans.

Ovariectomy results in cancellous osteopenia and accelerated bone turnover in the mouse. However, there are also clear differences between human and mouse physiology regarding the actions of estrogen and SERMs (60). While these species differences do not necessarily contraindicate its use, the ovariectomized mouse model should be approached with extreme caution.

Other Species

Rodents are generally not suitable as models for intracortical bone remodeling. Larger animals such as the dog are more appropriate for these studies because they have well developed Haversian remodeling. The dog, however, is not widely used as a model for postmenopausal osteoporosis. Whereas some investigators have detected bone loss following ovariectomy, with or without concurrent hysterectomy, other investigators have detected no changes (42). The relative insensitivity and inconsistent response of the dog skeleton to decreased gonadal hormones may be due to the long interval between periods of luteal activity. The same limitation may decrease the usefulness of common barnyard animals such as pigs and sheep as models for postmenopausal osteoporosis.

Several species of monkeys and apes have been investigated as models for postmenopausal bone loss. Monkeys and apes are generally more similar to human physiology

than either rodents or barnyard animals. Another advantage that monkeys and apes have over other large animals is the availability of molecular probes. Because of the species similarity, many human cDNA and RNA probes are suitable for use in monkeys. Ovariectomy results in bone loss in monkeys raised in captivity (21,22). However, recent studies in monkeys born in the wild have been less consistent. The use of monkeys and apes as a model for osteoporosis is greatly limited by their expense and limited availability.

ESTROGEN AS A FAMILY OF BONE ACTIVE COMPOUNDS WITH DISTINCT ACTIVITIES

Estrogen Metabolites

17 β -Estradiol is produced in the ovaries and acts as a hormone on peripheral target tissues. In contrast, the large family of estrogen metabolites which are structurally closely related to 17 β -estradiol are produced at many tissue sites. Unfortunately, only a small number of the individual estrogen metabolites have been evaluated regarding their skeletal effects.

Some of the complex and diverse effects generally attributed to 17 β -estradiol may actually be mediated by estrogen metabolites which are locally produced in target tissues. 17 β -Estradiol can be metabolized into an array of compounds by several enzymes, some of which may exist in isoforms. This exponential generation of metabolites may produce compounds which exert unique bioactivities. Furthermore, factors which influence the metabolism of 17 β -estradiol would have an impact on its bioavailability. Some of these metabolites, listed according to their enzymatic conversion, are described below.

17 β -ESTRADIOL INTERCONVERSION TO ESTRONE

Conversion is catalyzed by 17 β hydroxysteroid-dehydrogenase which is present in multiple isoforms. Each isoform has distinct requirements for substrates and cofactors, displays variable tissue distribution and subcellular localization, and may catalyze opposing reactions. The results are tissue-specific physiological responses. Thus, various factors which facilitate the oxidation of estradiol to estrone or its reduction back to estradiol indirectly influence the bioavailability of estradiol.

HYDROXYLATION

Although it is primarily catalyzed in the liver where there are many isoforms of NADPH-dependent cytochrome P450, there is evidence that hydroxylation also occurs at extrahepatic sites. The hydroxylated metabolites result from hydroxylation at C2, C4, and C16 positions. There are pronounced differences in the skeletal activities of these metabolites depending upon which position is hydroxylated.

METABOLIC CONJUGATION

Further metabolism by fatty acid acyl-CoA, sulfatases and β -glucuronidases can have two outcomes in estrogen-regulated pathways: 1) conjugation of aforementioned enzymes inactivates hormonal regulation and facilitates their ultimate excretion in the urine; 2) hydrolysis of conjugated metabolites, by enzymes at estrogen target tissues, provides a major contribution of extragonadal, bioactive estradiol at hormonally responsive sites. Conjugated equine estrogens isolated from urine contain delta 8,9-dehydroestrone sulfate which has been reported to have novel tissue selective actions in post-menopausal women (61).

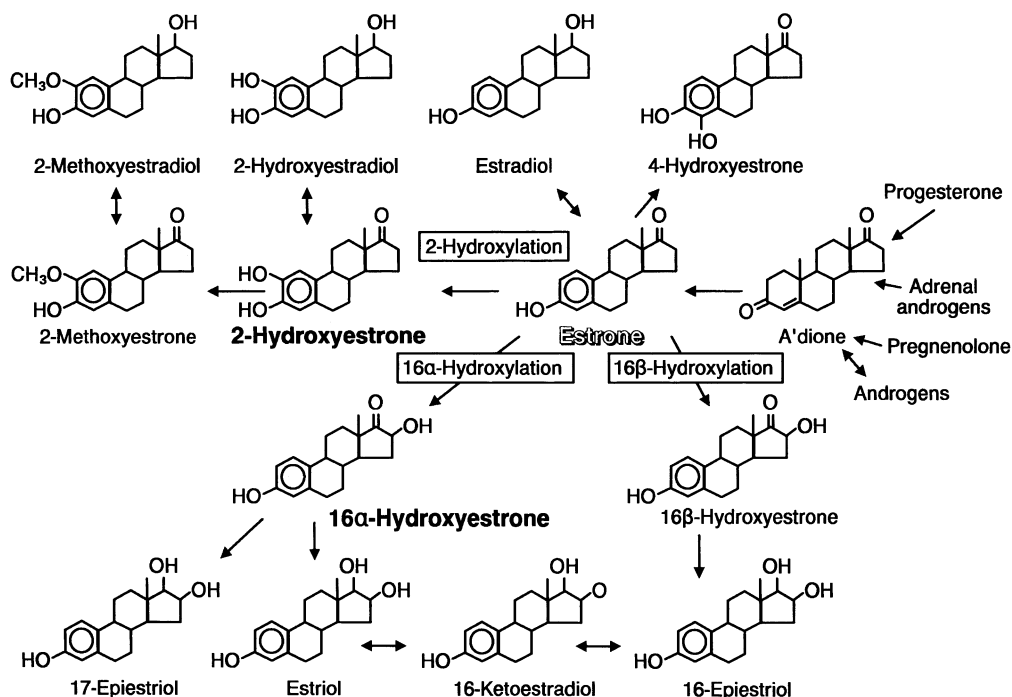


Fig. 1. Estrogen synthesis and metabolism. Estrone is synthesized at numerous extra ovarian sites and represents the most important locally produced estrogen. Hydroxylation at C2 and C16 represent the most important routes of metabolism of estrone.

METHYLATION OR ESTERIFICATION

While conjugation of hydroxylated metabolites of 17 β -estradiol and estrone makes them water soluble and facilitates their clearance, methylation by catechol-O-methyltransferase or esterification of monomethoxylated metabolites by acyl-coenzyme A, generates lipophilic metabolites, increases their half-life and reduces their binding affinity to the estrogen receptor. Methylation and esterification serve to inactivate estrogen receptor mediated activity but methylation at C2 confers novel activity.

Even though postmenopausal women lose bone at different rates, the levels of serum 17 β -estradiol do not differ among them (62). However, the serum levels of adrenal androgens and the conversion of these androgens and 17 β -estradiol to other estrogen metabolites do differ among individuals and certain races of the population and may play a role in maintaining bone mass in certain individuals.

The predominant estrogen in postmenopausal women, estrone is primarily metabolized through two mutually exclusive hydroxylation pathways (63,64) (Fig. 1). The catalytic conversion of estrone by the 2-hydroxylase or 16 α -hydroxylase enzymes results in the formation of either 2-hydroxyestrone or 16 α -hydroxyestrone, respectively. The 16 α -hydroxyestrone has been shown to bind covalently and noncovalently to the ER α , but with lower affinity compared to 17 β -estradiol. However, the metabolite's weak binding to the serum binding globulin makes it more available than the 17 β -estradiol for estrogen target tissues (65,66). 16 α -Hydroxyestrone was thought to be a complete estrogen agonist in reproductive tissues (65,66). More recent studies suggest that it can exhibit partial tissue selective activity (67). 2-Hydroxyestrone binds very weakly (even less than 16 α -

hydroxyestrone) to estrogen receptors, and has been shown to have no estrogenic activity on reproductive tissues and in some cases to act as a weak estrogen antagonist (68,69).

Lim et al. (70) reported that urinary levels of 16 α -hydroxyestrone were lower and 2-hydroxyestrone levels were higher in postmenopausal osteopenic individuals than in nonosteopenic individuals. Furthermore, the 16 α -hydroxyestrone/2-hydroxyestrone ratio was positively correlated with bone mineral density. African-American women, who are at lower risk for developing osteoporosis, reportedly have increased 16 α -hydroxylation and therefore have a higher ratio between the two metabolites compared to European-American women (71). When ovariectomized rats were treated with 16 α -hydroxyestrone, the cancellous bone turnover associated with ovariectomy was prevented in a manner similar to 17 β -estradiol (67). In contrast, 2-hydroxyestrone was inactive on bone (67). In agreement with these whole animal findings, 16 α -hydroxyestrone but not 2-hydroxyestrone, mimicked the effects of 17 β -estradiol on the regulation of alkaline phosphatase activity and osteocalcin secretion in an estrogen responsive osteoblast cell line stably expressing ER- α (72).

4-Hydroxyestrone is a minor metabolite of estrone which is a more potent carcinogen than the parent estrogen (73). 4-Hydroxyestrone is a less potent estrogen agonist on bone than 16 α -hydroxyestrone but is more potent than 2-hydroxyestrone (74).

Another naturally occurring estrogen metabolite which has been studied is 2-methoxyestradiol. 2-Methoxyestradiol is produced primarily by the liver and has very low affinity for estrogen receptors. 2-Methoxyestradiol has antitumorigenic activity and may act as a physiological tumor suppressor (73). Tumor cell proliferation is inhibited, possibly through the disruption of tubulin polymerization, and angiogenesis is reduced via increased senescence and apoptosis of endothelial cells (75). Osteosarcoma and other immortalized osteoblastic cells but not primary osteoblast cultures are killed by 2-methoxyestradiol by a nonestrogen receptor mediated mechanism (unpublished data). The administration of high concentrations of 2-methoxyestradiol to rapidly growing rats has been demonstrated to inhibit longitudinal bone growth but have no effect on either radial bone growth or cancellous bone turnover (76). This suggests that the metabolite reduces proliferation and/or stimulates apoptosis of rapidly dividing growth plate chondrocytes.

Estrogen was reported to have different effects on bone metabolism depending upon the route of administration to patients (77). The route of its administration has also been shown to influence the metabolism of 17 β -estradiol (78). Thus, it is possible that estrogen metabolites have an impact on the efficacy of hormone replacement therapy.

Phytoestrogens

Several classes of phytoestrogens have been identified. These include the isoflavones, the lignans, and the coumestans. Phytoestrogens are found principally in plants or their seeds, and a single dietary source often contains more than one class of phytoestrogens. Using a competition binding assay, Kuiper et al. (79) found that although the binding affinity of the phytoestrogens for either ER- α or ER- β was lower than that of 17 β -estradiol, the relative binding affinity of phytoestrogens for ER- β was significantly greater than for ER- α . In contrast to phytoestrogens, the relative binding affinity of the class of synthetic mixed estrogen agonists/antagonists referred to as selective estrogen receptor modulators (SERMs) which include tamoxifen and raloxifene was greater for ER- α than for ER- β . Using a reporter gene construct, the phytoestrogens tested were able to activate both estrogen receptor subtypes, although the overall potency of these compounds was much lower than 17 β -estradiol and many estrogen metabolites (79).

The most convincing evidence for phytoestrogen effects on bone comes from animal studies. To date, most studies have been in rats and have examined effects of various phytoestrogens on bone loss in ovariectomized rats, with generally positive findings. In addition to these animal studies, there are limited studies in postmenopausal women suggesting that phytoestrogens slow bone loss (80). Both genistein in mice and coumestrol in rats prevented postovariectomy bone loss by reducing bone resorption and osteoclast number in a manner very similar to 17β -estradiol (81,82). In contrast, other studies have demonstrated that the reduction of bone loss following ovariectomy in rats given genistein or soy protein was associated with unchanged parameters of bone resorption but increased bone formation rates (83–85). These data indicate that there may be important differences between the effects of phytoestrogens and 17β -estradiol on bone and, in fact, between different phytoestrogens. It has been suggested that some effects of phytoestrogens in different tissues may be due to estrogen receptor-independent mechanisms, i.e., through protein tyrosine kinases or other mechanisms (86).

It is important to note phytoestrogens have often been delivered to animals in a concentrated form using the sc route of administration. This is at least in part because phytoestrogens have limited bioavailability when consumed orally (87). Thus, it is not clear whether the observed effects can be achieved by consuming food containing phytoestrogens (88).

Selective Estrogen Receptor Modulators (SERMs)

Despite the success of hormone replacement therapy in the treatment of postmenopausal osteoporosis, prolonged treatment with estrogens even when supplemented with a progestin is associated with increased risk of breast and uterine cancer. Tamoxifen was the first estrogen receptor ligand recognized to possess tissue selective activity (8). Tamoxifen, a substituted triphenylethylene, was initially considered to be an antiestrogen based on its ability to antagonize estrogen mediated growth of normal uterus and mammary tumor cells. Tamoxifen's effectiveness at arresting breast cancer cell growth and low level of toxicity led to its widespread use as an adjunct defense in breast cancer therapy.

Tamoxifen slowed cancellous bone loss in ovariectomized rats and postmenopausal women but induced limited bone loss in ovary intact rats and premenopausal women (89). These findings indicate that tamoxifen is not a complete estrogen agonist on bone. Consequently, there has been a major effort to develop estrogen receptor ligands that further accentuate the desirable estrogen agonist properties in bone and the cardiovascular system without the undesirable growth-promoting effects in reproductive tissue. One such nonsteroidal analog, the benzothiophene derivative, raloxifene (LY139481 HC1), appears to satisfy several of these criteria in both laboratory animals and postmenopausal women.

Although raloxifene binds with similar affinity to ER- α and ER- β (90), the compound induces distinctly different conformational changes in ER- α and ER- β when it binds to the ligand-binding domain (91,92). Since the conformational differences affect the coregulatory factors recruited by each receptor, this provides a molecular explanation for the divergent effects of 17β -estradiol and raloxifene on gene transcription from various estrogen-inducible promoters (e.g., AP-1 sites) (93). These findings suggest that the tissue-selective responses to raloxifene and other partial estrogen agonists may be explained by a combination of the relative level of ER- α to ER- β , the repertoire of coregulators present in bone cells, and the type of DNA response element present in the promoter.

Table 2
Basic Research in SERM Ligands

<i>Compound name</i>	<i>Structural class</i>	<i>Comment</i>	<i>Reference</i>
Tamoxifen	Triphenylethylene	Approved treatment for breast cancer	119
Raloxifene	Benzothiophene	Approved for prevention of osteoporosis	118,123
Idoxifene	Triphenylethylene		134
Droloxifene	Triphenylethylene		122,128
Toremifene	Triphenylethylene		125,133, 136
FC1271a	Triphenylethylene		135
ICI 182,780	Steroid	Complete estrogen antagonist on reproductive tissues/mixed agonist/antagonist on other target tissues	58,130
EM-800/652	Nonsteroidal pro-SERM	"Highest affinity" estrogen receptor ligand	129,131, 132
LY compounds	Benzothiophene	Analogues of raloxifene with low energy conformation	120,121
Lasofloxifene	Triphenylethylene		126,127
MDL 103,323	Triphenylethylene		116,117

In the ovariectomized rat model of postmenopausal osteoporosis, raloxifene prevented bone loss from sites containing either cancellous or cortical bone, reduced serum cholesterol, but did not stimulate uterine hyperplasia (59,94,95). Interestingly, the effects in growing rats of 17 β -estradiol and raloxifene on bone growth and remodeling are not identical. Similar to 17 β -estradiol, raloxifene prevented the increases in longitudinal and radial bone growth as well as cancellous bone resorption that were induced by ovariectomy but, in contrast to the estrogen, did not prevent the ovariectomy-induced increase in cancellous bone formation (95). In adult rats with established osteopenia, raloxifene prevented additional bone loss but, as with estrogen, was unable to replace lost bone (40). The prevention of cancellous bone loss by raloxifene occurred by a mechanism mimicking the antiresorptive action of estrogen in which osteoclast number and eroded trabecular surfaces were reduced.

More recently, analogs of raloxifene which are more potent estrogen antagonists in the uterus have also been shown to prevent ovariectomy-induced bone loss when administered to rats immediately following ovariectomy; they also block continued bone loss when administered to osteopenic animals (96). In clinical trials of postmenopausal women, raloxifene reduced markers of bone resorption and formation and increased bone mineral density relative to patients receiving a placebo; effects were comparable to conjugated estrogens (97,98). Additionally, raloxifene therapy reduced the serum concentrations of total and low-density lipoprotein cholesterol but, unlike estrogen, did not stimulate the uterine endometrium.

Table 2 outlines some of the established and more recent SERMs developed, particularly those which exhibit promise as therapies to prevent osteoporosis. The table also references some reviews and seminal reports characterizing their actions. The "ideal SERM" remains to be developed. Nevertheless, these agents have been useful tools to elucidate mechanisms by which estrogen elicits a physiological response in bone as well as provide an alternative to estrogen for hormone replacement.

Table 3
Comparative Effects of Targeted Estrogen Receptor (ER) Gene Deletions
and Gonadectomy on Mouse Bone

Measurement	ER- α knockout		ER- β knockout		ER α/β knockout	
	Male	Female	Male	Female	Male	Female
Cortical bone mass	↓	↓	NC	↑	↓	↓
Bone elongation	↓	NC	NC	↑	↓	?
Cancellous bone mass	NC	NC	NC	↑	NC	↓

↓, Decreased; ↑, increased; NC, no change; ?, unknown.

MECHANISMS OF ACTION OF ESTROGEN ON BONE

Estrogen Receptor Mediated Pathways

GENE DELETION OF ESTROGEN RECEPTORS

The development of mice exhibiting targeted gene deletions (knockout) in either the ER- α , ER- β , or both genes simultaneously, has been achieved. The results of these studies were recently reviewed in detail (2) and are summarized in Table 3. As a note of caution, deletion of estrogen receptors from all cells could affect bone indirectly as a result of alterations in other estrogen target tissues. In this regard, disturbing estrogen receptor signaling in all tissues by estrogen receptor isoform gene ablation may destabilize the feedback loops regulating synthesis of other sex steroids in addition to estrogens, thereby leading to changes in the circulating levels of these hormones as well as cytokines regulated by these hormones, some of which also possess major osteotropic activity. For example, the ER- α knockout female mouse has been reported to have markedly elevated levels of 17 β -estradiol whereas the male ER α knockout has increased levels of testosterone (99). 17 β -Estradiol and testosterone regulate the synthesis of IGF-I, an important mediator of bone cell differentiation and activity. As a further complexity, phenotypes in single estrogen receptor gene mutants may arise as a result of the β -isoform being able or unable to either compensate for the loss of the α -isoform or, alternatively, suppress ER- α activity. In the latter case, loss of ER- β could conceivably enhance rather than suppress estrogen responsiveness in a cell type that normally expresses both estrogen receptor isoforms.

Overall, it is becoming clear that ER- α and ER- β perform different functions in cortical and cancellous bone and that the relative importance of the two isoforms at these sites differs between the sexes. However, it is questionable whether the estrogen receptor isoforms perform identical functions during skeletal growth and turnover in humans. Perhaps the most surprising result arising from the estrogen receptor knockout analysis is that the double ER- α/β knockout mice are viable with grossly normal skeletons, demonstrating that both estrogen receptor isoforms are dispensable for normal development of cartilage and bone. Further, the skeletal effects of ER- α/β knockout in mice differ from gonadectomy. Future studies in which ER α and ER β are deleted selectively from bone cells might help clarify the physiological role of the receptors in bone metabolism.

ESTROGEN REGULATION OF CYTOKINE SYNTHESIS IN OSTEOBLASTS

In bone, a hallmark of estrogen deficiency is an elevated rate of bone turnover in which both osteoclast-mediated bone resorption and, osteoblast-mediated bone formation/

activity occur at an increased rate. There is evidence that estrogens action on bone proceeds through a cascade (1,2). The release of cytokines such as IL-1, TNF, M-CSF, GM-CSF, IL-6, and IGF-I, is an important component of the cascade mechanism, and is responsible for the accelerated bone turnover. The role of growth factors in mediating the actions of estrogen on bone metabolism have been reviewed. Briefly, the osteoblast and/or monocyte/macrophage lineage release of numerous cytokines that are capable of increasing osteoclast formation, differentiation or activity has been shown to be inhibited by 17β -estradiol. However, which of the cytokines are most important for mediating the actions of estrogens remains unresolved.

Elegant knockout and receptor blockade studies have shown that IL-1, IL-6, and TNF- α are important for estrogen action on bone. However, these studies do not distinguish between permissive and regulatory roles for these cytokines. The only cytokine demonstrated to be regulated by estrogen in skeletal tissues in vivo is IGF-I, whose expression is down regulated by the hormone (10,39).

Other factors such as osteoprotegerin (OPG), a soluble member of the TNF receptor family, may also mediate the inhibitory effect of estrogens on bone resorption. OPG binds to and inhibits the activity of OPG ligand (OPGL or RANKL), a TNF-related cytokine produced by bone marrow stromal cells and osteoblasts (among other cell types) which has been shown to be essential for osteoclast formation. Among the bone-active cytokines, OPG is unique because deletion of the OPG gene in mice generates severe osteopenia in both cancellous and cortical bone (100), whereas mice over-expressing OPG are osteopetrotic (101). 17β -Estradiol has been shown to increase OPG expression in osteoblasts overexpressing the ER- α gene (102), and this effect may be indirect via TGF- β which has been shown to increase OPG production by bone marrow stromal osteoblast precursor cells (103). If these effects also occur in vivo, OPG may be a key mediator of the anti-resorptive effects of estrogens.

MOLECULAR MECHANISM OF ESTROGEN REGULATION OF CYTOKINE AND OTHER GENE PROMOTERS

The molecular mechanisms involved in the estrogen regulation of several cytokine gene promoters have been studied. Many of these promoters lack consensus estrogen response elements (EREs) and thus have revealed other mechanisms of transcriptional control by estrogen receptors which in some instances do not require direct binding of the ligand-activated estrogen receptors to DNA. Instead, estrogen receptors interact with other transcription factors and co-regulators to modulate the binding affinity of these proteins to their cognate regulatory sites in the DNA. For example, repression of the IL-6 promoter by estrogen occurs because protein-protein interactions between the ligand-activated ER- α and the transcription factors NF- κ B and C/EBP β prevent binding of the latter factors to their respective sites on DNA (104,105). In contrast, estrogen can stimulate transcription from the IGF-I promoter via enhanced binding of *fos/jun* heterodimers to AP-1 elements through interaction with activated ER- α (106). Numerous genes including retinoic acid receptor- α 1 (RAR- α 1), *c-fos* proto-oncogene, cathepsin D, IGF binding protein-4 and the epidermal growth factor receptor gene, among others, are modulated by estrogens through the binding of ER-Sp1 transcription factor complexes with either Sp1 sites alone or Sp1/ERE half sites (107–109). However, as a note of caution, estrogen increases IGF-I mRNA levels in osteoblastic cells in culture but decreases the expression of this cytokine in several skeletal tissues in vivo suggesting that much remains to be learned regarding the molecular mechanisms of the hormone's action.

Alternative Mechanisms

The cascade model explains many apparently nongenomic actions of steroids which are, in fact, genomic processes. However, there is a growing body of evidence that steroids also influence the cell surface by nongenomic effects which involve responses to steroids within minutes and are too rapid to be explained by genomic processes (110,111). For instance, a possible cardioprotective effect of estrogen has been shown to occur through the induction of endothelial nitric oxide synthase in human vascular endothelial cells via a ligand-dependent interaction between ER- α and phosphatidylinositol-3-OH kinase (112). In osteoblastic cells in culture, treatment with 17 β -estradiol has been shown to cause a rapid (within seconds) increase in intracellular calcium ion concentration due to influx across the plasma membrane and release from intracellular stores (113). Additionally, estrogen stimulates hydrolysis of phosphatidylinositol phospholipids with the generation of diacylglycerol and inositol 1,4,5-triphosphate which in turn activate other second messenger pathways. In osteoblasts from female rats this effect of the hormone has been shown to be mediated by activation of phospholipase C- β 2 via a pertussin toxin-sensitive G protein's β subunits, further supporting the existence of a nonclassical membrane estrogen receptor (114). Activation of MAP kinases and increases in cAMP and cGMP have also been reported in osteosarcoma cells (115). The response of the target cell to estrogens may therefore be a superimposition of genomic and nongenomic effects, although the physiological importance of the nongenomic effects is still unclear. The putative membrane receptors for steroid hormones remain unidentified.

SUMMARY

Estrogen is not essential for the growth and differentiation of bone and cartilage cells. However, estrogen is important to the age-related changes in bone mass and architecture as well as successful adaptation to the increased mineral requirements during reproduction. Changes in the levels of circulating estrogens contribute to the sexual dimorphism of the skeleton, creation and retention of cancellous bone during growth in excess of that required to fulfill the mechanical requirements of the skeleton, mobilization of this reservoir during pregnancy and lactation, and extensive bone loss following menopause, predisposing women to osteoporosis. Estrogen influences bone metabolism by regulating the rate of production, activity and lifespan of bone and cartilage cells.

Estrogen influences bone metabolism through direct and indirect actions on bone cells. Many of the direct actions are mediated through the estrogen receptors that are present in the target cells but evidence also exists for alternative mechanisms.

17 β -Estradiol has the highest affinity to estrogen receptors of any natural ligand and functions as a hormone. Estrogen metabolites are produced locally and bind to estrogen receptors with lower affinity. The level of estrogen agonism and tissue selectivity is dependent upon the molecular structure of the metabolite. Additionally, some estrogen metabolites have activities which differ from 17 β -estradiol and may be mediated through alternative pathways.

Estrone and its metabolites are the principal estrogens in postmenopausal women. The circulating and tissue levels of these metabolites may play a role in the rate and severity of postmenopausal bone loss.

The mechanisms of action of estrogens on the skeleton are incompletely understood. An improved understanding of the actions of estrogen on bone metabolism and mecha-

nisms which mediate these actions is likely to lead to the development of improved therapies to prevent the detrimental changes which follow menopause.

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Clinical Trials of Estrogen and SERMs on Bone Markers, Bone Mineral Density, and Fractures

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ESTROGEN AND OSTEOPOROSIS

Osteoporosis is the most prevalent metabolic bone disease in developed countries, including the United States. Osteoporosis-associated fractures increase with age, and with the aging population, it is estimated that osteoporosis will reach epidemic proportions. In postmenopausal Caucasian women in the United States, 54% have osteopenia and 30% have osteoporosis (1). The estimated cost of hip fractures alone in the United States could reach \$240 billion within 50 yr (2). Mortality after fracture is high, reaching 24% after hip fracture. However, the most feared consequences of an osteoporosis-related fracture are the morbidity, associated loss of independence and impaired quality of life (3).

Early cross-sectional studies revealed a decrease in bone mineral density associated with menopause. Women who undergo premature menopause have a lower bone density in the spine and femur compared to age-matched controls (6). Once estradiol levels fall below 40–80 pg/mL, bone loss can be detected. In postmenopausal women there is a

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rapid phase of bone loss of about 3%/yr in the spine that lasts for approx 5 yr after the menopause (4). Subsequently, bone loss slows down to approx 0.5%/yr. The major mechanism for the rapid phase of bone loss is estrogen deficiency. Estrogen deficiency increases activation frequency and causes a remodeling imbalance. The administration of estrogen and progestogens can prevent the initial rapid bone loss. Although the exact mechanism of estrogen on bone is unclear, there are many possibilities. Estrogen may work through the osteoblast causing increased synthesis of osteogenic substances like insulin-like growth factor and transforming growth factor- β .

The major action of estrogen on the skeleton is an inhibition of bone resorption via actions on osteoclasts. Evidence in avian, rabbit, and human species suggests a direct action on the osteoclast by regulation of specific genes. Estrogen also induces apoptosis, reducing the life span of the osteoclast. The synergy of several cytokines such as IL-6, IL-1, IL-2, tumor necrosis factor- α , and others enhance osteoclast recruitment, differentiation, and activity. A reduction in the production of these cytokines by estrogen prevents bone resorption. One of the most potent inhibitors of osteoclastogenesis is osteoprotegerin (OPG). The natural ligand for OPG is related to the cytokine TNF and identical to TRANCE/RANKL, a factor that enhances T-cell growth and function of dendritic cells. OPG production is increased by estrogen and this effect may explain much of the protection provided to the skeleton by estrogen (5).

Estrogens may also improve calcium balance by increasing intestinal calcium absorption either by a direct effect on the gut and indirectly by stimulation of 1,25-dihydroxyvitamin D production. There may be increased tubular resorption of calcium in the kidney through direct action on the renal tubule or via stimulation of parathyroid hormone secretion.

PROBLEMS WITH CLINICAL STUDIES WITH ESTROGEN REPLACEMENT THERAPY

Many published studies using HRT/ERT are uncontrolled, and many of those that contain a control group have inherent flaws. Control groups consist of reference populations, calcium-treated controls, or parallel controls. Most available randomized studies comparing the effects of estrogen and placebo on bone are of a relatively short duration (2 yr or less) and long-term effectiveness (over 10 yr, for example) is unknown.

RANDOMIZED CONTROLLED CLINICAL TRIALS: THE POSTMENOPAUSAL ESTROGEN/ PROGESTIN INTERVENTIONS TRIAL (PEPI)

Estimates of the percentage of women who lose bone while taking postmenopausal hormone replacement therapy (HRT) vary greatly and range from 10–30%. Difficulties arise in assessing the correct bone loss as within-person measurement error must be considered in longitudinal measurements. Women who do not have an increase in BMD in response to HRT use are dubbed “nonresponders.” In a recent analysis from the Postmenopausal Estrogen/Progestin Interventions (PEPI) Trial, the frequency of bone loss among women using HRT was assessed. A total of 875 healthy early postmenopausal women aged 45–64 yr were recruited at seven clinical centers. Treatments included placebo, conjugated equine estrogen (CEE), 0.625 mg/d; CEE, 0.625 mg/d plus medroxyprogesterone acetate (MPA), 10 mg/d for 12 d/mo; CEE, 0.625 mg/d plus MPA,

2.5 mg/d; or CEE, 0.625 mg/d plus micronized progesterone (MP), 200 mg/d for 12 d/mo. Replicate BMD measures were used to calculate within-person measurement errors. In this study, the investigators developed several cutpoints (percentage loss criteria) to classify subjects who were losing bone. This approach was adopted because there is no universal agreement on the standard that defines bone loss in an individual subject. In the first 12 mo, 1.5% of HRT users lost BMD at the lumbar spine and only 0.6% of treated women lost BMD at the spine in months 12–36. At the total hip, 2.3% of women who were adherent to HRT lost BMD in the first 12 mo but only 0.4% were classified as losing bone at the hip in months 12–36. The authors concluded that in the first 3 yr after therapy is begun bone loss in women taking HRT is rare. The study also implies that the first 12-mo follow-up BMD may not be an accurate assessment of response to treatment in postmenopausal women (7).

Estrogen and Bone Markers

Until the publication of the PEPI trial, there were few large randomized, placebo-controlled trials evaluating the effect of estrogen on biochemical markers of bone remodeling. This 3-yr trial also evaluated the effect of estrogen on eight bone turnover markers and how changes in marker levels were related to bone mineral density of the spine and hip in postmenopausal women. The association of bone turnover markers with baseline and 1 yr changes in the lumbar spine and hip bone mineral density were examined in 293 subjects. In the active treatment groups, the mean levels of all markers decreased and remained below baseline values throughout the three years of study. In the 54 women assigned to the placebo group there was no change from baseline in the mean level of any marker. At baseline, age and body mass index accounted for 16% and 25% of the variance in hip and spine BMD, respectively. Excretion of the resorption marker, N-telopeptide of type 1 collagen (NTX) alone accounted for 12% and 8% of variance at the spine and hip, respectively, while excretion of the carboxy-terminal telopeptide of type 1 collagen (Crosslaps) accounted for 8% and 7% of variance, respectively. However, changes in marker levels were not related to change in BMD. The authors concluded that the bone turnover markers are not an adequate surrogate for BMD to identify women with low bone mass and provided little information for predicting BMD changes for individual untreated or HRT-treated postmenopausal women (8).

BMD in the PEPI Trial

Bone mineral densities at baseline, 12 and 36 mo were the main outcome measures for PEPI. Participants assigned to the placebo group lost an average of 1.8 and 1.7% of the spine and hip BMD at the end of three years. Those assigned to active treatment gained BMD at both sites ranging from 3.5 to 5.0% in spinal BMD and a total increase of 1.7% in hip BMD. Women assigned to CEE plus continuous (MPA) had significantly greater increases in spinal BMD than those assigned to the other three active regimens (average increase 5.0 vs 3.8%, respectively). However, among adherent participants, there were no significant differences in BMD changes among the four active treatment groups. When stratified to evaluate older women, women with low initial BMD and those with no previous hormone use, it was shown that all three groups gained significantly more bone than younger women, women with higher initial BMD or those who used hormones previously. Thus, this study indicated that estrogen replacement therapy increased BMD at clinically relevant sites (9).

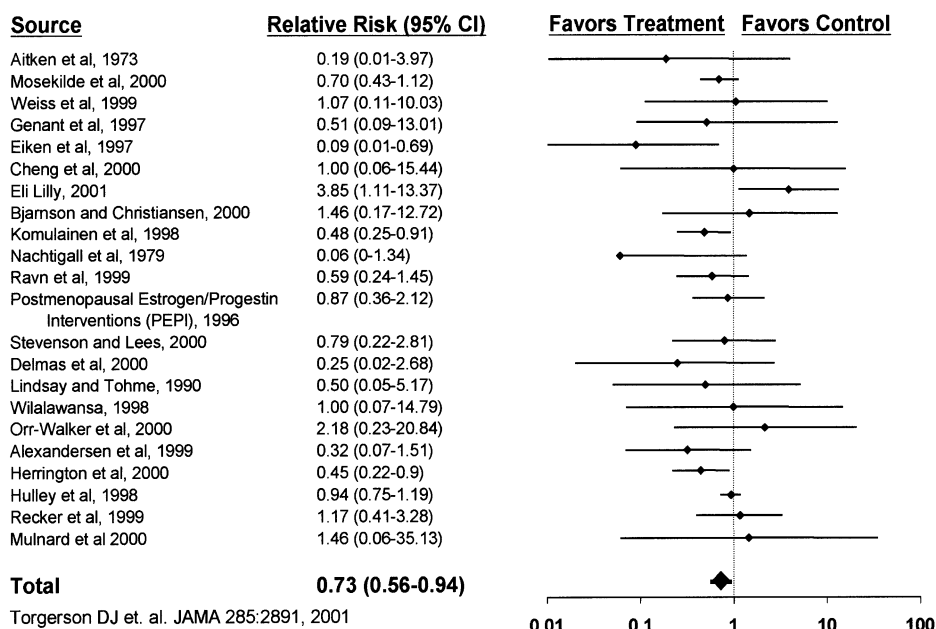


Fig. 1. Meta-analysis of fracture risk in trials utilizing HRT. Adapted from ref. 14.

Efficacy of oral and transdermal estrogens appear similar, and in a randomized, placebo-controlled trial of transdermal estrogens, bone mineral density of the spine increased from 0.8 to 3.7% (10). Transcutaneous estrogen gel has also been shown to have a 3% increase in BMD of the spine when compared to placebo (11).

Several other interesting results have been derived from the PEPI Trial. For instance, ascorbic acid is a required cofactor in the hydroxylations of lysine and proline necessary for collagen formation. Using the PEPI Trial data, the cross-sectional relationship between dietary vitamin C intake and bone mineral density was assessed. After adjustment for age, BMI, estrogen use, smoking, leisure/physical activity, calcium and total energy intake, each 100 mg increment in dietary vitamin C intake was associated with a 0.017 g/cm² increment in BMD ($p = 0.002$ femoral neck; $p = 0.005$ total hip). There was a similar association of the use of vitamin C with lumbar spine BMD, but after adjustment this was finding not statistically significant (12).

HRT AND FRACTURE PREVENTION

Numerous observational studies have suggested that HRT prevents osteoporotic-related fractures. However, the evidence from prospective trials is limited. Inherent problems with the available randomized, controlled trials leaves considerable uncertainty concerning the magnitude of a protective effect of HRT in the prevention of osteoporotic fractures. Most of the trials have insufficient subject numbers or are of insufficient length to adequately assess fracture risk. In one study that utilized a preparation of transdermal estrogen (0.1 mg 17 β -estradiol days 1–21 and oral medroxyprogesterone acetate days 11–21), eight new fractures occurred in seven women in the treatment group compared to 20 fractures in 12 placebo-treated women (13).

In a recently published meta-analysis, all trials of estrogen that were more than 1 yr in length were analysed for fracture prevention. There was a 27% reduction in

nonvertebral fractures in a pooled analysis. The relative risk of nonvertebral fracture with estrogen use was 0.73 (95% CI 0.46–0.98) with a reduced effect in women greater than 60 years of age (RR 0.88; 95% CI 0.71–1.08). The effect of estrogen to specifically prevent hip and wrist fractures was more pronounced with a RR of 0.60 (95% CI 0.40–0.91) (Fig. 1) (14). In a meta-analysis performed with the expertise of the Cochrane group, 56 studies met predetermined criteria for inclusion that randomized postmenopausal women to HRT or control (placebo or calcium/vitamin D) of at least one year duration. In this analysis, HRT showed a trend toward a reduced incidence of vertebral fractures and nonvertebral fractures (15).

HRT AND COMPLIANCE

Life expectancy in Western countries is up to age 80 yr for women. The average age of menopause is close to age 50 suggesting that approx 30 yr of a woman's life is in an "estrogen deficiency" state. In spite of observational evidence for fracture prevention and randomized, controlled clinical trial evidence for preservation of BMD with estrogen, only a small number of at-risk women are willing to take estrogen. There are numerous reasons why patients discontinue medications in general, but estrogen compliance is particularly problematic.

Use of estrogen in the postmenopausal woman clearly has bone benefits, but, as with many pharmacologic interventions, is not without attendant risks. Since unopposed estrogen is associated with an increased risk of endometrial hyperplasia and cancer, it is rarely prescribed without concurrent administration of a progestogen to attenuate these risks. The risk of breast cancer in women taking estrogen remains controversial. Although many studies do not indicate an increased breast cancer risk with estrogen replacement therapy (16), other studies suggest an increased risk that may be associated with a greater dose and/or duration of use or with the progestogen utilized (17). A slight increase in the risk of thromboembolic events is also noted in estrogen users. Due to these various side effects and other liabilities, long-term compliance for hormone replacement therapy (HRT) use is poor. Up to 50% of women discontinue therapy within months, and approx 10% use the medication only sporadically. Twenty-three percent never fill their prescription, and at the end of three years, only 20% continue to take HRT (18).

The PEPI Trial is one of the few large randomized, placebo-controlled trials evaluating the use of hormone replacement therapy in early postmenopausal women. One interesting analysis was performed to evaluate how many patients continue hormone replacement therapy after the end of the 3-yr intervention when hormonal side effects have frequently subsided. Among the women who were assigned to a treatment group that included estrogen, continued post-PEPI hormone use was more common in women who used hormones before the PEPI Trial and in women who were adherent to hormone use during the trial. Women who had undergone a hysterectomy were also more likely to continue hormone use. Older age, less education, and being non-Caucasian predicted less hormone use. Post-PEPI hormone use was also affected by geography as use was highest in San Diego and lowest in Iowa City. Women who were on placebo and lost more bone mineral density were more likely to begin hormones after the trial than women with less bone loss. Lipids, blood pressure, and other cardiovascular risk factors had relatively little influence on hormone use. Thus, the main predictors of post-PEPI hormone use were education, ethnicity, geographic region, hysterectomy and prior use/adherence (19). Several new approaches to estrogen use are being evaluated including using lower doses to attenuate side effects.

LOW-DOSE ESTROGEN THERAPY FOR PREVENTION OF BONE LOSS

For many years it was believed that the lowest effective dose of estrogen replacement therapy is 0.625 mg/d of conjugated estrogen or its equivalent of 1.0 mg of estradiol. In fact, lower doses of HRT have not been extensively tested for efficacy in preventing bone loss, particularly in older patients. Early studies of lower doses were often problematic because vitamin D and calcium intake may not have been adequate, and there are several more recent analyses that suggest lower doses of estrogen might be of benefit. For instance, in the Study of Osteoporotic Fractures levels of estradiol >5 pg/mL were associated with an OR of 0.4 for vertebral or hip fracture. In subjects with estradiol levels <5 pg/mL, there was a 30% increase in hip fractures and a 23% increase in vertebral fractures (20). Studies in men also indicate that very low amounts of estrogen could provide some protection from bone loss (21).

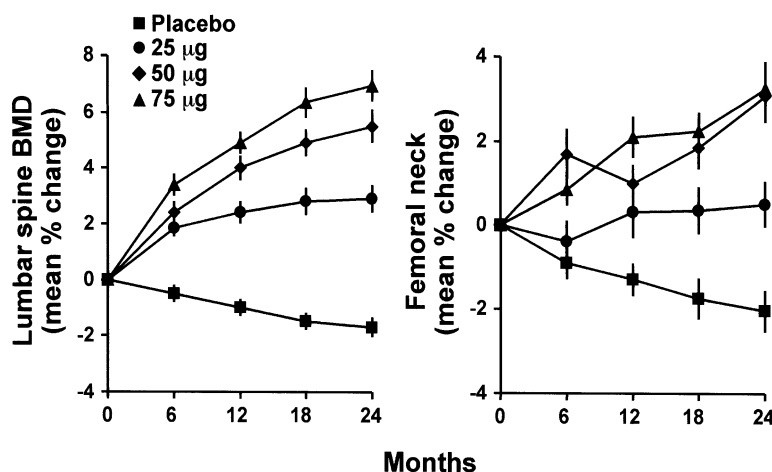
Lower doses of estrogen have recently been evaluated in clinical trials, especially in older patients. As early as 1987, Ettinger et al. found that 0.3 mg of conjugated equine estrogen (CEE), when combined with calcium administration, prevented bone loss compared to placebo in early postmenopausal women. There was some bias in the study design, as patients were allowed to select their treatment, in turn possibly enhancing compliance rates. HRT was provided as CEE 0.3 mg on days 1–25 with MPA, 10 mg, or norethindrone, 5 mg on days 16–25. The annual rate of BMD loss in untreated postmenopausal women was 9% in the lumbar spine as measured by quantitative computed tomography. In women treated with the combination of calcium and low-dose estrogen, trabecular spinal mineral content increased 2.3% (22).

Transdermal 17 β -estradiol has also been evaluated at lower doses in several studies. In one randomized, double blind, dose-ranging, placebo-controlled study, Cooper and colleagues evaluated the effect of 25, 50, and 75 μ g/d of transdermal 17 β -estradiol on bone mineral density (Fig. 2). All women received oral dydrogesterone 10 mg/d (with estrogen) or placebo. Calcium intake was maintained at 1000 mg/d. A clear dose response was shown with greater increases in BMD at the lumbar spine noted with higher doses of transdermal 17 β -estradiol. An increase in BMD was seen in the group treated with only 25 μ g/d. At the femoral neck, no difference was found between the 75 and 50 μ g doses, and both were greater than the placebo group (23).

Similar findings were found with low-dose esterified estrogen therapy (25). Women were randomly assigned to receive unopposed continuous esterified estrogens (0.3, 0.626, or 1.25 mg/d) or placebo for 2 yr (Fig. 3). All doses of esterified estrogens produced increases in BMD of the lumbar spine compared with baseline values and with placebo at 6, 12, 18, and 24 mo of therapy. This study also measured estradiol levels in response to therapy and correlated this information with BMD results. Plasma estradiol concentrations of approx 90 pmol/L or greater were associated with positive BMD changes from baseline ($p = 0.001$) (Fig. 4) (25). These results are similar to those of Ettinger et al. (20) who noted increased BMD in untreated women if endogenous estradiol levels were >36 pmol/mL. The minimum level of plasma estradiol believed to prevent bone loss had previously been considered to be 180–220 pmol/mL (24).

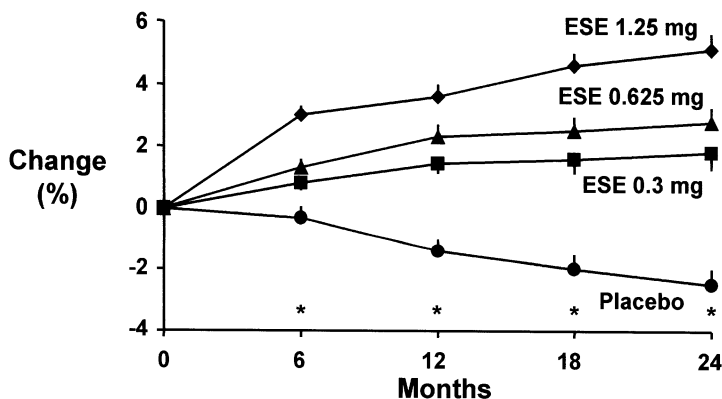
ESTROGEN THERAPY IN OLDER WOMEN

Women over the age of 70 are at the greatest risk for osteoporotic fracture. Although several studies suggest that estrogen replacement therapy will decrease bone turnover



Cooper C et al: Osteoporosis Int 9:358, 1999

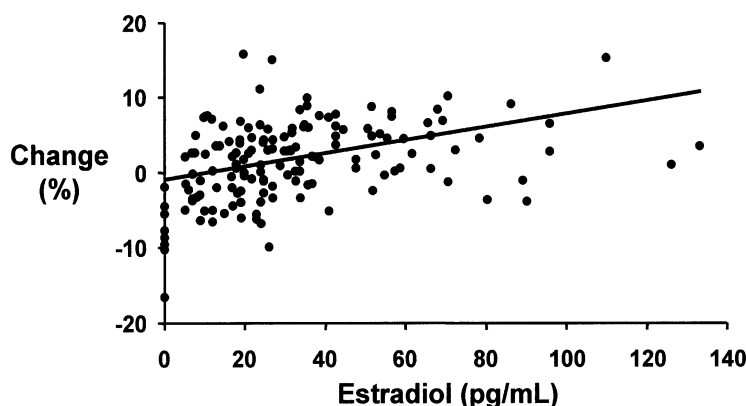
Fig. 2. Effect of low-dose transdermal estradiol on lumbar spine and femoral neck BMD. A total of 277 early postmenopausal women were enrolled in this placebo-controlled, two-year study. Three different dosages (25, 50, and 75 µg/d) were combined with sequential oral dydrogesterone (20 mg/d) to prevent bone loss. At the end of two years, lumbar spine BMD as assessed by DEXA was increased $4.7\% \pm 0.7\%$ with estradiol (closed circles, 25 µg/d), $7.3\% \pm 0.7\%$ with estradiol (diamonds, 50 µg/d), and $8.7\% \pm 0.7\%$ with estradiol (triangles, 75 µg/d). There were also significant increases in the femoral neck, trochanter, and total hip BMD at all doses of estradiol compared with placebo (23).



*All doses of ESE significantly different from placebo, $P < 0.05$
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Fig. 3. Mean percentage change from baseline of lumbar spine BMD measured by DEXA in postmenopausal women treated with unopposed esterified estrogen (0.3 (squares), 0.625 (triangles) or 1.25 mg/d (diamonds) or placebo (circles). BMD changes at all times and at all esterified estrogen doses were significantly different from those occurring with placebo ($P < 0.05$) (25).

and slow bone loss, few studies have examined the effect of estrogen on the older population. In one example, 31 healthy women over 70 yr of age were randomized to 12 wk of treatment with either micronized 17β -estradiol (0.5 mg/d) or 1500 mg/d elemental calcium provided as calcium carbonate plus vitamin D (800 IU/d) (26). At the end of the initial 12-wk treatment, both groups received calcium, vitamin D and estradiol for an additional 12 wk. Eleven patients were followed for 36 wk without any treatment and



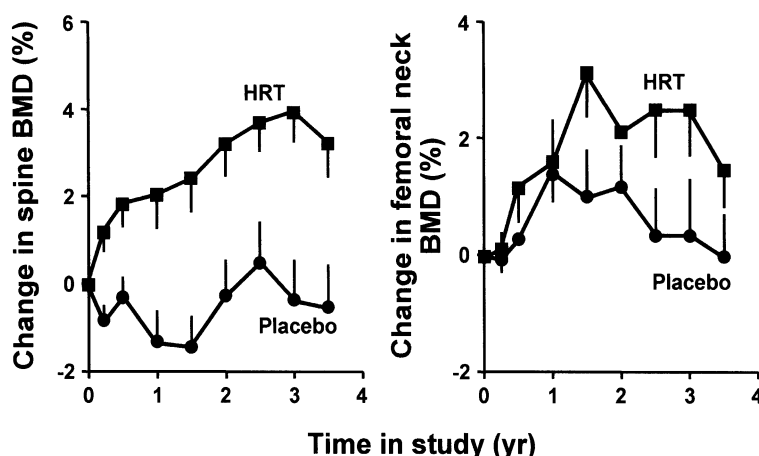
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Fig. 4. Postmenopausal women ($n = 406$) were randomly assigned to receive continuous esterified estrogen (0.3, 0.625, or 1.25 mg/d) or placebo for 24 mo. In this figure, individual subject plasma concentrations of estradiol were correlated with lumbar spine BMD changes from baseline ($P = 0.001$) (25).

served as a control group. Markers of bone resorption (urinary cross-linked C-telopeptide and N-telopeptide and type I collagen, serum cross-linked N-telopeptide of type I collagen, urinary free deoxypyridinoline cross-links, and serum bone sialoprotein) all decreased with initial treatment and decreased further with combination therapy ($p < 0.001$). Markers of bone formation (bone alkaline phosphatase, osteocalcin, and type I procollagen peptide) decreased with calcium and D treatment, but not with estradiol alone. There were no additional effects of combination therapy on bone formation markers compared to the group taking calcium and vitamin D alone. This initial report suggests that there was an additive effect of low-dose estrogen and calcium on bone resorption but not on bone formation in older women.

Other investigators have considered the effect of continuous low-dose estrogen and progesterone combined with calcium and vitamin D in older women. Healthy caucasian women greater than age 65 yr with low bone mass were recruited and randomized to continuous therapy with a relatively low dose of conjugated equine estrogen, (0.3 mg/d, plus medroxyprogesterone, 2.5 mg/d, or matching placebo) (Fig. 5) (27). All received calcium supplements to bring calcium intakes to about 1000 mg/d and supplemental oral 25-hydroxyvitamin D was provided to maintain serum 25-hydroxyvitamin D levels of at least 75 nmol/L in both groups. Over the 3.5 yr of the trial, bone mineral density increased by 3.5% in the intention-to-treat analysis and by 5.2% among patients with >90% adherence to therapy. Femoral neck bone density in the HRT group increased steadily for 1.5 yr, and then decreased by the end of 3.5 yr. In general, the effect of therapy on BMD did not reach statistical significance although values were higher in the HRT group than in the placebo group at every time point. If analyses are performed using only data from subjects with 90% adherence to the treatment, there is almost 5% gain in BMD for the spine, 2.5% for total body BMD and approx 1.6% for the hip (27).

Several studies have evaluated higher doses of estrogen in the older patient, sometimes in combination with other treatments. These studies have debunked the myth that older patients, with lower bone turnover, may not benefit from intervention with estro-



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Fig. 5. Low-dose continuous conjugated equine estrogen and medroxyprogesterone acetate compared to placebo in healthy Caucasian women. In this study, 128 women older than 65 yr of age were randomized to receive continuous conjugated equine estrogen, 0.3 mg/d and medroxyprogesterone, 2.5 mg/d (square) while the others received identical-appearing placebo pills (circles). Average age was 73 yr at the time of entry. Percentage change in bone mineral density of the lumbar spine and femoral neck are shown with the HRT group represented by squares and the placebo group by circles. The effect of HRT at these doses was significant at all sites except for the femoral neck (27).

gen therapy. In a recently published trial, the effects of estrogen and 1,25 dihydroxy-vitamin D therapy, provided individually or in combination, on bone loss in elderly women were described. These women, aged 65–77 yr, had normal bone density for their age and were entered into a randomized, double-blind, placebo-controlled trial that included four treatment groups: conjugated equine estrogens (ERT) (0.625 mg/d) given to women who had had a hysterectomy; medroxyprogesterone acetate (2.5 mg/d) was added to ERT in women who had an intact uterus (HRT); calcitriol alone (0.25 µg bid); or a combination of HRT or ERT plus calcitriol. Over a 3-yr period, HRT/ERT produced an increase in BMD of 2.98% at the femoral neck and 4.36% at the spine. Calcitriol alone increased BMD 0.10% at the femoral neck and 1.65% at the spine. Combination therapy (HRT/ERT plus calcitriol) increased BMD 3.80% at the femoral neck compared to 4.91% at the spine. All three treatment groups differed from placebo at the spine. There were no differences between combination therapy and HRT/ERT alone on BMD at any site when using an intention-to-treat analysis. If the analysis was performed only using data from women who were adherent to treatment, calcitriol had a greater effect on spine and total hip. In this group of women, HRT/ERT alone and in combination with calcitriol therapy was highly effective to reduce bone resorption and increase BMD at the hip and other clinically relevant sites. Significant bleeding and spotting (defined as >70 episodes) were common events. Bleeding occurred in 27% of patients within the first year, 4 and 3%, respectively in the second and third years. Twenty percent of estrogen treated women experienced only spotting in the first year, 16% in the second year and 8% in the third year. Episodes of hypercalciuria occurred in 8% of patients on placebo, 26% on calcitriol, 3.3% on estrogen and 14.8% on the combination. Thus, side effects associated with estrogen therapy were common in this older population, particularly in the initial

months. Bleeding led to discontinuation in 11% of women and 5% of women discontinued treatment due to breast tenderness (28).

A study conducted through the Washington University Older American's Independence Center evaluated the effects of estrogen replacement in frail elderly women (29). This randomized, double-blind, placebo-controlled trial evaluated the effects of HRT on BMD in women aged 75 yr or older with mild-to-moderate physical frailty. Physical frailty was defined as meeting two or three criteria: low-peak aerobic power (VO_{2peak}) of 11–18 mL/min/kg of body weight, self-reported difficulty or need for assistance with two instrumental activities of daily living (ADL) or one basic ADL, or modified physical performance test score of 18–22. For 9 mo, subjects were provided conjugated estrogens, 0.625 mg/d, and cyclic medroxyprogesterone acetate (MPA), 5 mg/d for 13 consecutive days every third month. Patients without a uterus were not provided MPA or placebo MPA. Treatment adherence was assessed with pill counts. BMD of the total body, lumbar spine, and proximal femur was measured at 3-mo intervals using DEXA. Patients who are unable to tolerate prescribed estrogen due to adverse effects (7 HRT, 2 placebo) had the dosage reduced to 0.625 mg every other day for 1–4 wk, and then dosage was increased to 0.625 daily. Those that continued to have adverse effects returned to 3–4 tablets/wk for the remainder of the study period. Based on intention-to-treat analyses, increases in BMD at total body, lumbar spine, total hip and trochanter measurement sites were larger in response to HRT compared to placebo treatment. The increases in BMD were also larger in the subset of women who were 80% adherent to treatment compared to all women randomized to HRT. The between group difference in women adherent to HRT compared to placebo treatment at the femoral neck was 2.6%. With the intention-to-treat analysis, there was a between group difference (treatment vs placebo) of 3.9% in the lumbar spine and of 1.4% in the femoral neck. Differences were also noted in BMD at the total body, lumbar spine, total hip, and trochanter. This study provides novel information on the skeletal response to the initiation of HRT in physically frail elderly women. Elderly women may have a positive response because of the effects of estrogens on calcium conservation in extraskelatal organs. Estrogen preserves intestinal responsiveness to vitamin D, increases parathyroid hormone independent tubular reabsorption of calcium, and decreases PTH secretion during hypocalcemia. Of course, other potentially beneficial effects of estrogen include suppressive effects on bone resorbing cytokines associated with chronic inflammation and aging (29).

“ULTRA”-LOW-DOSE ESTROGEN THERAPY

Ultra low doses of parenteral 17β -estradiol have been provided to elderly women who were 60 yr or older for 6 mo, in the form of a vaginal ring that provided 7.5 μ g/24 h. Forearm bone density increased by 2.1% in estradiol users, in contrast with a decrease in nonusers of –2.7%. Markers of bone formation (bone specific alkaline phosphatase and osteocalcin) decreased in the treatment group suggesting reduced bone turnover. Studies of this type are useful since poor compliance is one of the major issues with long-term hormone replacement therapy at higher doses, especially in the older age group. Although this was a short-term study, there was no evidence of endometrial proliferation as determined by ultrasonography during six months of exposure in two additional studies using the same treatment regimen (30). Further work is needed to determine the minimum effective dose of estrogen to prevent bone loss (see section on Low-Dose Estrogen for review of additional studies in this population).

THE USE OF SERMS FOR POSTMENOPAUSAL OSTEOPOROSIS

Although estrogen replacement therapy is frequently prescribed for treatment of menopausal symptoms and the possible prevention of long term health problems related to the menopause, compliance is low due to adverse effects. In a recent telephone survey of women members of the Kaiser Foundation Health Plan, 62% of those aged 50–55 and 48% of women 65 yr or older discontinued HRT within 1 yr of starting treatment. Treatment-related side effects were the most frequent reasons for therapy discontinuation, especially vaginal bleeding (52% of women >65 yr and 29% of women aged 50–55 yr) (31). The availability of new pharmaceutical agents with mixed estrogen agonist and antagonist properties has allowed a subset of women to enjoy skeletal benefits while avoiding common adverse effects of estrogen.

Selective estrogen modulators or SERMs are used for the treatment of postmenopausal osteoporosis or as adjuvant treatment for breast cancer. In order to understand how a compound such as a SERM can have mixed agonist and antagonist effects at a particular target tissue, a review of how the estrogen receptor binds to its ligand is helpful. The ligand, in this case estradiol, binds to and activates a receptor. The receptor binds to tissue-specific gene regulating elements and activates transcription within the cell nucleus, thereby resulting in gene activation. Within the limits of this simple model, it has been difficult to understand how the SERM compounds can have distinct actions in specific tissues. However, the estrogen receptor, like other steroid receptors, also binds auxiliary proteins that enhance or inhibit its activity. The binding of estradiol determines the configuration of the receptor-ligand complex and in turn determines which activator or inhibitor proteins are bound. Thus, the effect on the target tissue may depend on the activator/inhibitor proteins that are available in the target tissue, and on the structural configuration of the ligand-receptor complex. When the estrogen receptor binds to a SERM, the receptor-ligand complex has a configuration different than that of the estradiol-receptor combination. In its new configuration, a different set of activators (or inhibitors) may be turned on, and genes are affected differently. Thus, a SERM, through its unique conformational changes, can appear to have an antagonistic effect on a tissue (e.g., the uterus), where estrogen has an agonist effect. Add to this degree of complexity the recent discovery of more than one estrogen receptor, (ER α and ER β), and the possible combinations of receptor conformations and subsequent gene activation rapidly multiply.

In summary, there are multiple steps in the cell pathway where a ligand such as estradiol or one of the SERMs can exert tissue-specific effects: binding to one of two estrogen receptors (α vs β), altered affinity of binding to activation of a repressor or activator protein in the target tissue, changes in the receptor-ligand conformation, and activation of diverse and specific gene sequences (32–34).

FIRST GENERATION SERMS: TAMOXIFEN

Triphenylethylene compounds were evaluated in the 1940s for their effects on various tissues. These early compounds were disappointing as antitumor agents, and it was not until 1969 that tamoxifen, a compound with a greatly improved toxicity profile, was introduced. Tamoxifen is a partial estrogen agonist, but also has some properties as an estrogen antagonist (35). Tamoxifen binds to estrogen receptors and in some tissue blocks the effect of endogenous estrogens. Importantly, tamoxifen antagonizes the growth of estrogen-dependent breast tumor cells and is used in breast cancer patients as

an adjuvant therapy to surgical mastectomy. Tamoxifen has been shown to reduce the risk of breast cancer in premenopausal women as reported in 1998 by the National Cancer Institute (NCI)/National Surgical Adjuvant Breast and Bowel Projects' Breast Cancer Prevention Trial. In a 5-yr study of 13,388 high-risk women, tamoxifen reduced the risk of breast cancer by 45% (37). The FDA has approved tamoxifen for the prevention of breast cancer in high-risk individuals. Interim analysis of smaller, less powerful European-based trial of tamoxifen for the prevention of breast cancer indicated no protective effect of the drug (38).

Several studies have indicated that tamoxifen may also act as a bone antagonist in estrogen-replete premenopausal women. A subset of the National Cancer Institute-sponsored trial participants had BMD measurements at baseline, 1, 2, and 5 yr. The trial was stopped early due to the success of the primary endpoint, so the number of participants with BMD measurements at the 5-yr mark is reduced. The effects of tamoxifen on bone and mineral metabolism in premenopausal women was surprising, since the opposite effect was noted in postmenopausal women.

Most studies with tamoxifen suggest it has a partial protective effect on the skeleton in postmenopausal women. Tamoxifen has been shown to prevent bone loss in ovariectomized rats, confirming this estrogen-like effect on bone. Studies by Love et al. indicated that postmenopausal women taking tamoxifen as an adjuvant therapy for breast cancer, had less bone loss than a placebo-treated control group (36).

Tamoxifen also has estrogen-like effects on some elements of the cardiovascular system. Tamoxifen increases high density lipoprotein cholesterol and decreases low density lipoprotein cholesterol in breast cancer patients. Venous thrombosis is a side effect of tamoxifen as it is with estrogen. The effect of tamoxifen on the uterus has only recently been appreciated and with long-term use, endometrial hyperplasia with subsequent risk for endometrial cancer has been described. The most common adverse symptoms of tamoxifen therapy include hot flashes, vaginal discharge, and irregular menses.

Currently tamoxifen is utilized as adjuvant therapy for prevention of breast cancer recurrence. The usual dose is 10 mg/d twice a day for up to 5 yr. In a patient with an intact uterus, monitoring with yearly vaginal ultrasound or endometrial biopsy to detect endometrial hyperplasia remains controversial and consensus is not imminent. Alternatively, some clinicians utilize a progestogen to antagonize the endometrium in a manner similar to traditional HRT. Based on limited data, monitoring of BMD in premenopausal women using tamoxifen is warranted. In postmenopausal women, the guidelines suggested for HRT could be followed with the understanding that small positive changes in BMD are likely to result from tamoxifen use.

THE EFFECTS OF RALOXIFENE ON BONE METABOLISM

The ability of raloxifene to maintain bone density in animal studies lead to the development and implementation of clinical testing. Markers of bone turnover are decreased by the administration of raloxifene in postmenopausal women, but to a lesser extent than with the administration of conjugated equine estrogens. A double-blind, randomized controlled study compared the effects of raloxifene (60 mg/d) to those of conjugated equine estrogen (0.625 mg/d) (39). There was a marked decrease in CTX (53%) and NTX (43%) in the estrogen-treated women compared with a decrease of 23 and 22%, respectively, in raloxifene-treated women. Osteocalcin, bone specific alkaline phosphatase,

and C-terminal type I procollagen peptide were measured as markers of bone formation, and reductions in these measures were also greater in the estrogen-treated group. In this same study, bone mineral density increased in both raloxifene and estrogen treatment groups within the 6-mo study period, but the group of women treated with conjugated equine estrogens had a greater increase. This head-to-head trial suggested that raloxifene had smaller effects on bone turnover and bone density than conjugated equine estrogen. Bone biopsies were obtained in this study and histomorphometric analysis revealed no significant differences in static indices at baseline. Dynamic histomorphometric indices of bone remodeling (BFR/BV, BFR/BS, and Ac.F) were decreased from baseline to the 6-mo endpoint in the conjugated equine estrogen group, but not in the raloxifene group. Mineralizing surface (MS/BS) and MAR did not change in either treatment group suggesting that the mineralization was not adversely affected. The authors noted that bone architecture was similar in the two treatment groups, with comparable trabecular number and thickness.

In another study, three doses of raloxifene (or placebo) were administered to 601 postmenopausal women (Fig. 6). Women who received placebo had a decrease in their bone mineral density. At the end of two years, the group receiving raloxifene (60 mg/d) had a 2.4% increase in the BMD of the lumbar spine compared to those treated with placebo, and a 2.4% increase at the total hip. Each of the groups receiving raloxifene (30, 60, or 150 mg) had a decrease in bone markers including serum osteocalcin, bone alkaline phosphatase and urinary type I collagen C-telopeptide (CTX) in comparison with placebo. At the 60-mg dose, all bone turnover markers decreased to premenopausal levels (40).

The Multiple Outcomes of Raloxifene Evaluation (MORE) study was a multicenter, randomized, blinded, placebo-controlled trial with 7,705 participants age 31–80 yr. Women were at least 2 yr postmenopausal and had a BMD T-score of ≤ -2.5 . Participants were randomized to 60 mg/d or 120 mg/d of raloxifene or placebo. The main outcome measurements included vertebral fracture, nonvertebral fractures, and bone mineral density. Assessment of bone markers was performed and the mean baseline serum osteocalcin concentration was 24.1 mg/L and urinary excretion of C-telopeptide was 248 mg/mmol of creatinine. Serum osteocalcin concentrations decreased by a median 8.6, 26.3, and 31.1% after 36 mo in the placebo, 60 mg and 120 mg raloxifene, respectively. Urinary C-telopeptide excretion decreased to a similar amount (8.1, 34.0, and 31.5% in placebo, 60 mg and 120 mg of raloxifene, respectively). In this trial, vertebral fracture was defined as a decrease in the anterior, mid or posterior vertebral height of at least 20% and at least 4 mm. Baseline and follow-up radiographs were available for 89% of the women. Women receiving raloxifene had fewer new vertebral fractures regardless if they had existing fractures at the beginning of the study. For the 60 mg/d raloxifene group, the relative risk (RR) of vertebral fracture was 0.7 (95% confidence interval [CI] 0.5–0.8) and for the 120 mg/d group, the RR was 0.5 (CI, 0.4–0.7). Recently, the MORE cohort has been followed for 4 yr and the reduction of vertebral fracture risk was maintained. The relative risk in the 60 mg raloxifene-treated group was 0.64 compared with placebo (41). The overall occurrence of nonvertebral fractures was low and although there was a 10% reduction with raloxifene, it did not reach a level of significance (RR 0.9; 95% CI 0.1–1.1). Bone mineral density increased compared to placebo at the femoral neck (2.1%) and the spine (2.6%) at the 60 mg/d dose ($p < 0.001$). One of the major

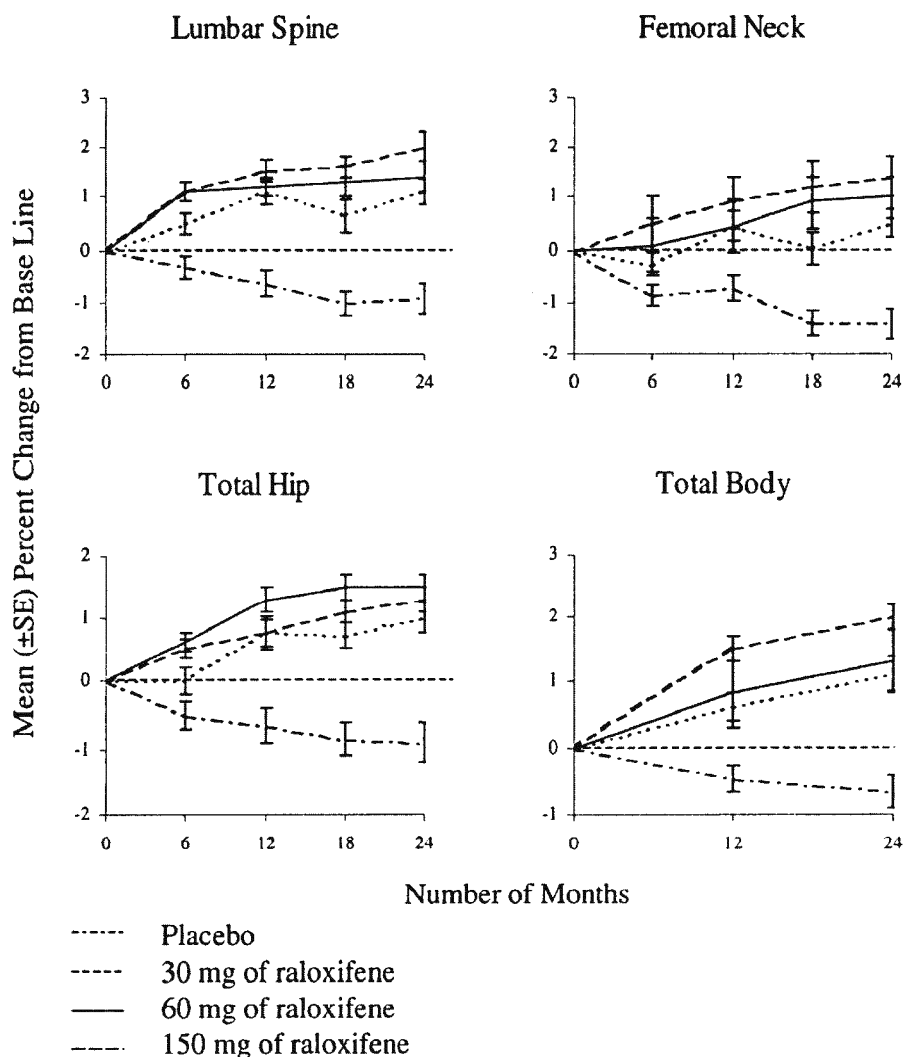


Fig. 6. Data from the two-year time point in the multiple outcomes of raloxifene study (MORE). There is an increase in BMD as determined by DEXA as expressed by the mean percent change from baseline at the doses of raloxifene tested as compared to placebo (40).

side effects was that women receiving raloxifene had an increased risk of venous thromboembolism versus placebo (RR, 3.1; 95% CI, 1.5–6.2). Other side effects included a slight increase in the number of hot flashes in the raloxifene-treated patients (6.4% in the placebo group vs 9.7% in the 60 mg/d of raloxifene) and an increase in leg cramp (placebo 3.7% vs 7.0% at 60 mg/d raloxifene) (42).

In a joint effort, European and North American study groups enrolled a total of 1,145 healthy, postmenopausal women in parallel, double-blind, randomized, placebo-controlled trials of identical design. The women were randomly assigned to receive raloxifene (30, 60, or 150 mg) or placebo daily for 3 yr. The change in lumbar spine BMD in the placebo group was –1.32%. Lumbar spine BMD increased 0.71% in the 30 mg raloxifene group, 1.28% in the 60 mg group and 1.20% in the 120 mg group. Similar changes in BMD were observed at the hip. Serum low-density lipoprotein cholesterol

was reduced 7–12% below baseline in the raloxifene-treated patients. As in the MORE trial, the only significant adverse effect was hot flashes (25% in the 60 mg raloxifene group compared to 18% in the placebo group) (43).

EFFECTS OF OTHER SERMS ON PARAMETERS OF BONE METABOLISM

Levormeloxifene is a selective estrogen receptor modulator that was thought to possess desirable effects on the skeleton and cardiovascular system without inducing endometrial hyperplasia. In a phase II, 12-mo interim analysis of a 2-yr multicenter, double-blind, placebo-controlled study, the effect of levormeloxifene on bone mineral density, biochemical markers, lipid profile and endometrial safety was assessed in 301 postmenopausal women. Participants were between 45 and 65 yr of age, at least 1 yr postmenopausal and had an intact uterus. The trial included women both with normal and low BMD. Subjects were randomized to levormeloxifene 1.25, 5, 10, or 20 mg, or continuous combined HRT in the form of 17 β -estradiol (1 mg) and norethisterone acetate (NETA, 0.5 mg/d) or placebo. Bone resorption, as reflected by serum C-terminal telopeptide levels, decreased by 50% in the levormeloxifene groups. There was no dose-response effect noted. Levels in the HRT-treated group decreased more than 60% and in the placebo group, which received 500 mg of calcium alone, decreased by about 10%. The pattern was similar for bone-specific alkaline phosphatase with slightly less suppression in all levormeloxifene groups compared to HRT (30% for levormeloxifene group and 50% for the HRT group). Serum osteocalcin also decreased in both levormeloxifene and HRT groups. Pronounced changes were noted in the lumbar spine BMD measurements. BMD fell by less than 1% in the placebo group and increased by about 2% in the levormeloxifene group and by almost 5% in the HRT group at the end of 1 yr. Similar changes were seen in measures of total hip and total body BMD.

Unfortunately, the effects on the endometrium resulted in a cessation of further clinical development of levormeloxifene. Endometrial thickness increased in the levormeloxifene group while there was no difference in endometrial thickness between HRT and placebo groups. At the point of the interim analysis (1 yr), the endometrial thickness caused no discomfort and no dropouts. Hot flashes did not occur more frequently in the levormeloxifene group than in the placebo group, but occurred less frequently in the HRT group. In addition, eight women, all in the levormeloxifene group, experienced uterovaginal prolapse during the trial. Although levormeloxifene did not induce endometrial hyperplasia or significant endometrial proliferation, the increase in endometrial thickness was thought to be related to fluid accumulation and may have caused the uterovaginal prolapse (44).

Other SERMs have been in clinical development but effects on the endometrium have hindered further testing. These include droloxifene and idoxifene. Both of these SERMs appear to have estrogen agonist effects on bone. Undoubtedly, the development of SERMs will continue and it is likely that compounds with beneficial as well as selective effects will be identified.

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Androgen Action in Bone

Basic Cellular and Molecular Aspects

Kristine M. Wiren, PhD

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INTRODUCTION

The obvious impact of the menopause on skeletal health has focused much of the research on the general action of gonadal steroids on bone on the specific effects of estrogen. However, androgens, independently, have important beneficial effects on skeletal development and on the maintenance of bone mass in both men and women. For example, androgens (1) influence growth plate maturation and closure helping to determine longitudinal bone growth during development, (2) mediate dichotomous regulation of cancellous and cortical bone mass, leading to a sexually dimorphic skeleton, (3) modulate peak bone mass acquisition, and (4) inhibit bone loss (1–4). In castrate animals, replacement with nonaromatizable androgens (e.g., dihydrotestosterone) yields beneficial effects that are clearly distinct from those observed with estrogen replacement (5,6).

In intact females, blockade of the androgen receptor (AR) with the specific AR antagonist hydroxyflutamide results in osteopenia (7). Data suggest that combination therapy with both estrogen and androgenic steroids is more effective than estrogen replacement alone (8–11). At the same time, nonaromatizable androgen alone and in combination with estrogen also result in distinct changes in bone mineral density in females (12). These reports illustrate the independent actions of androgens and estrogens on the skeleton. Thus, in both men and women it is probable that androgens and estrogens each have important, yet distinct, functions during bone development and in the subsequent maintenance of skeletal homeostasis. Given the increasing awareness of the importance of the effects of androgen on skeletal homeostasis, and the potential use of androgen for the treatment of bone disorders, much is yet to be learned.

The mechanism(s) by which androgens affect skeletal homeostasis are thus the focus of intensified research. Androgen receptors have been identified in a variety of cells found in bone tissue (13). The characterization of androgen receptor expression in these cells thus clearly identifies bone as a target tissue for androgen. The direct actions of androgen to influence the complex processes of proliferation, differentiation, mineralization, and gene expression in the osteoblast have also been documented (14). Androgen effects on bone cells may also be indirectly modulated and/or mediated by other autocrine and paracrine factors in the bone microenvironment. This chapter will thus review recent progress on the characterization of androgen action in bone cells.

MOLECULAR MECHANISMS OF ANDROGEN ACTION IN BONE CELLS: THE ANDROGEN RECEPTOR

A steroid hormone target tissue can be defined as one that possesses both functional levels of the steroid receptor and a measurable biological response in the presence of the hormone. As described in this chapter, bone tissue clearly meets this standard with respect to androgen. Direct characterization of AR expression in a variety of tissues, including bone, was made possible by the cloning of the androgen receptor cDNA (15,16). Colvard et al. (17) first described the presence of AR mRNA and specific androgen binding sites in normal human osteoblastic cells. This report characterized the abundance of both androgen and estrogen receptor proteins as similar in osteoblasts (Fig. 1), suggesting that androgens and estrogens may each play important roles in skeletal physiology. Subsequent reports have confirmed AR mRNA expression and/or the presence of androgen-binding sites in both normal and clonal, immortalized or transformed osteoblastic cells, derived from a variety of species (18–23). The size of the AR mRNA transcript in osteoblasts (about 10 kb) is similar to that described in prostate and other tissues (15), as is the size of the AR protein analyzed by Western blotting (~110 kDa) (22). There is a report of two isoforms of AR protein in human osteoblast-like cells (~110 and ~97 kDa) (24) similar to that observed in human prostate (25). Whether these isoforms possess similar functional activities in bone, when expressed at similar levels as described in other tissues (26) is yet to be determined.

The number of specific androgen-binding sites in osteoblasts varies, depending on methodology and the cell source, from 1000–14000 sites/cell (21,22,24,27), but is in a range seen in other androgen target tissues. Furthermore, the binding affinity of the AR found in osteoblastic cells ($K_d = 0.5\text{--}2 \times 10^{-9}$) is typical of that found in other tissues. Androgen binding is specific, without significant competition by estrogen, progester-

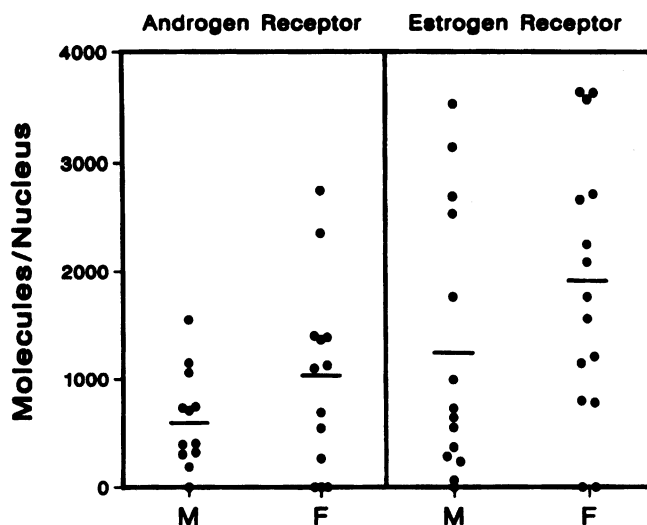


Fig. 1. Nuclear androgen and estrogen receptor binding in normal human osteoblast-like cells. Dots represent the mean calculated number of molecules per cell nucleus for each cell strain. (Left) Specific nuclear binding of [3 H]R1881 (methyltrienolone, an androgen analog) in 12 strains from normal men and 13 strains from normal women. (Right) Specific nuclear [3 H]estradiol binding in 15 strains from men and 15 strains from women. The horizontal lines indicate the mean receptor concentrations (17).

one, or dexamethasone (17,22,24). Finally, testosterone and dihydrotestosterone (DHT) appear to have similar binding affinities (18,22). All these data are consistent with the notion that the direct biologic effects of androgenic steroids in osteoblasts are mediated at least in part via classic mechanisms associated with AR signaling.

THE ANDROGEN RECEPTOR SIGNALING PATHWAY

The AR is a member of the class I (so-called classical or steroid) nuclear receptor superfamily, as are the estrogen receptor, progesterone receptor, mineralocorticoid and glucocorticoid receptor (28). These steroid receptors are ligand-inducible transcription factors with a highly conserved modular design consisting of transactivation, DNA binding and ligand binding domains. Cellular localization of the AR in the absence of ligand is somewhat controversial. Unliganded AR has been reportedly found both predominantly in the cytoplasmic compartment (29–31), or alternatively, predominantly in the nucleus in a large complex of molecular chaperonins, consisting of loosely bound heat-shock and other accessory proteins (20). Being lipid molecules, androgenic steroids can diffuse freely through the plasma membrane to bind the AR. Once bound by ligand, the AR is activated and released from this protein complex. As shown in Fig. 2, this allows the formation of homodimers (or potentially heterodimers) that bind to DNA at palindromic androgen response elements (AREs) in androgen responsive gene promoters. The ARE sequences are found characteristically as a motif represented by an inverted repeat separated by 3 bp (32) similar to glucocorticoid response elements (33), but androgen-specific regulation at nonconventional direct repeat AREs has also been shown (34). DNA binding of the activated AR organizes a cascade of events in the nucleus leading to transcription and translation of a specific network of genes that is responsible for the cellular response to the steroid (35). In the classic model of steroid

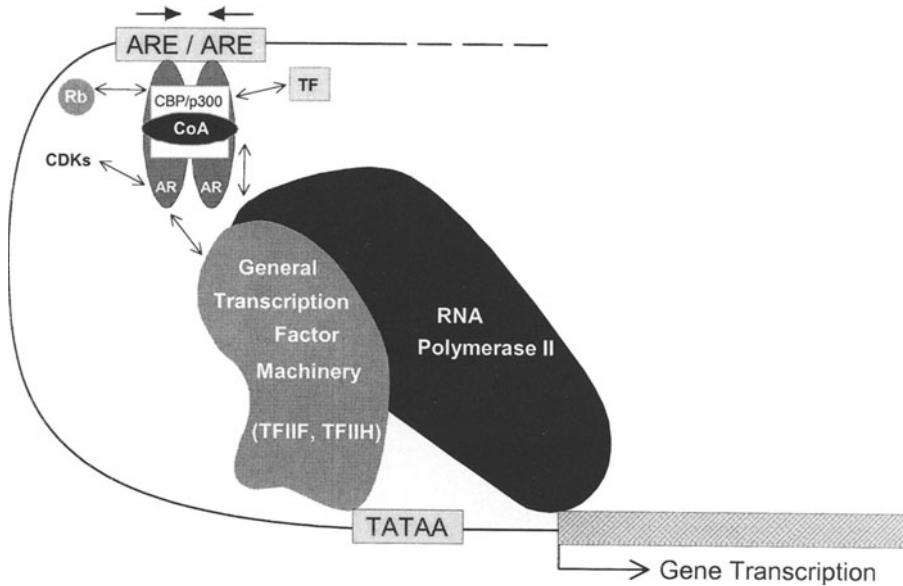


Fig. 2. Model of AR regulation of gene expression. Binding of androgen promotes high-affinity dimerization, followed by DNA binding at the androgen response element (ARE) in an androgen-responsive gene promoter. Coactivators may remodel chromatin through histone acetylase activity to open chromatin structure (*141*), or act as a bridge to attract TFs that target binding of TATA-binding protein to the TATAA sequence (*142*). The retinoblastoma tumor suppressor product also activates AR transactivation (*143*). Phosphorylation of receptor may result from activation of phosphorylation cascades, such as by cyclin-dependent kinases (CDKs). AR can also directly contact TFIIF (*144*) and TFIIF (*145*) in the general transcription machinery. Such interactions between the AR and the general transcription machinery, leading to stable assembly, results in recruitment of RNA polymerase II and subsequent increased gene transcription.

action, the latent receptor is converted into a transcriptionally active form by simple ligand binding. This model is now considered an over-simplification, with the understanding that signaling pathways and additional proteins (for example, coactivators or corepressors as described below and shown in Fig. 2) within the cell can influence steroid receptor transduction activity. For example, steroid receptor phosphorylation can result from signal transduction cascades initiated at the cell membrane, for example with cyclin-dependent kinases (*36*). It has been shown that steroid receptor phosphorylation can lead to alteration of the responsiveness of steroid receptors to cognate ligands or, in some cases, even result in ligand-independent activation.

Such potential modification(s) of AR action in bone cells is in fact only poorly characterized; whether the AR in osteoblasts undergoes post-translational processing that might thus influence receptor signaling (stabilization, phosphorylation, etc.) as described for AR in other tissues (*37,38*), and the potential functional implications of such modifications (*39*), are not known. Ligand-independent activation of AR by cellular phosphorylation cascades has been described in other tissues (*40,41*), but has not been explored in bone. AR activity may also be influenced by receptor modulators, such as the nuclear receptor coactivators or corepressors (*42,43*). As outlined in Fig. 2, these coactivators/corepressors can influence the downstream signaling of nuclear receptors through multiple mechanisms, including histone acetylation/deacetylation, respectively,

that results in chromatin remodeling. Such activities may reflect both the cellular context and the particular promoter involved. In addition, a recent report documents direct acetylation of the AR itself by p300/CBP (44). AR specific coactivators have been identified (45), many of which interact with the ligand binding domain of the receptor (46). Expression and regulation of these modulators may thus influence the ability of steroid receptors to regulate gene expression in bone (47), but this has been underexplored with respect to androgen action. A preliminary report has suggested the presence of androgen-specific coactivators in osteoblastic cells (48).

In addition to the classical AR pathway present in bone cells, several other androgen-dependent signaling pathways have been described. Specific binding sites for weaker adrenal androgens (dehydroepiandrosterone [DHEA]) have been described (49,50), raising the possibility that DHEA or similar androgenic compounds may also have direct effects in bone cells. In fact, Bodine et al. (51) showed that DHEA caused a rapid inhibition of *c-fos* expression in human osteoblastic cells that was more robust than that seen with classical androgens (dihydrotestosterone (DHT), testosterone, or androstenedione). Nevertheless, all androgenic compounds significantly increased transforming growth factor-beta (TGF- β) activity in osteoblastic cells. Androgens may also be specifically bound in osteoblastic cells by a 63-kDa cytosolic protein (52). There are reports of distinct AR polymorphisms identified in different races that may have biological impact on androgen responses (53), but this has not been explored with respect to bone tissue. These different AR isoforms have the potential to interact in distinct fashions with other signaling molecules, such as c-Jun (54). Androgens may also regulate osteoblast activity via rapid nongenomic mechanisms through elevations in intracellular calcium levels (55,56) mediated by receptors at the bone cell surface (57), as has also been shown for estrogen (58). Finally, AR may also interact with other transcription factors, such as NF- κ B, CREB-binding protein and different forms of AP-1, to generally repress transcription without DNA binding (59,60). The role and biologic significance of these nonclassical signaling pathways in androgen-mediated responses in bone are still relatively uncharacterized.

LOCALIZATION OF ANDROGEN RECEPTOR EXPRESSION

Clues about the potential sequelae of AR signaling may be derived from a better understanding of the cell types in which receptor expression is documented. In the bone microenvironment, the localization of AR expression in osteoblasts has been described in intact human bone by using immunocytochemical techniques (13,30). In developing bone from young adults, Abu et al. (13) showed ARs were predominantly expressed in active osteoblasts at sites of bone formation (Fig. 3). ARs were also observed in osteocytes embedded in the bone matrix. Importantly, both the pattern of AR distribution and the level of expression was similar in males and in females. Furthermore, AR was also observed within the bone marrow in mononuclear cells and endothelial cells of blood vessels. Expression of the AR has also been characterized in cultured osteoblastic cell populations isolated from bone biopsy specimens, determined at both the mRNA level and by binding analysis (24). Expression varied according to the skeletal site of origin and age of the donor of the cultured osteoblastic cells. AR expression was higher at cortical and intramembranous bone sites and lower in cancellous bone; this distribution pattern correlated with androgen responsiveness. AR expression was highest in osteoblastic cultures generated from young adults and somewhat lower in samples from either

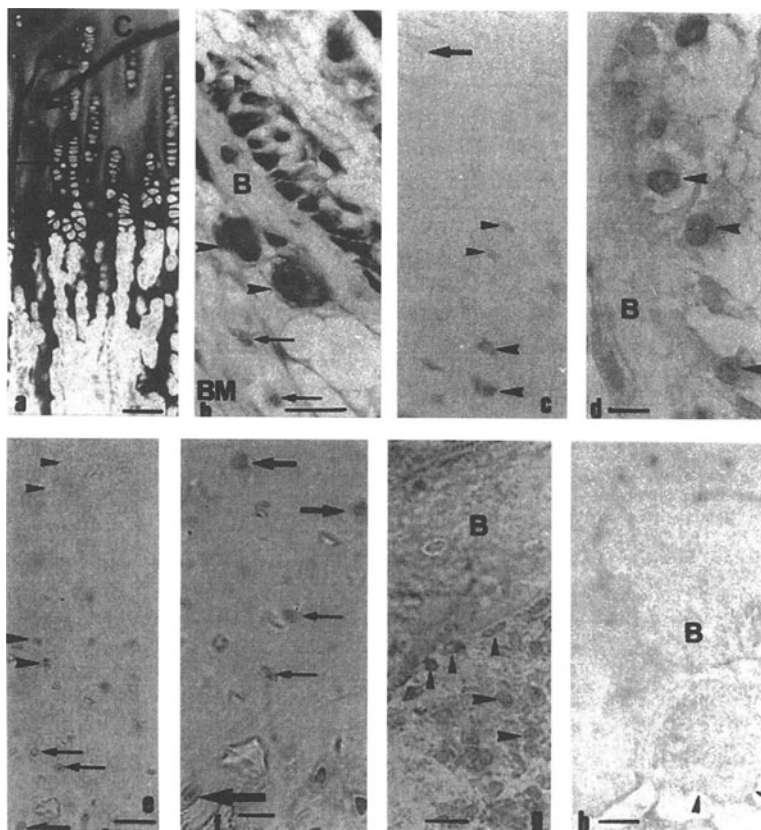


Fig. 3. The localization of AR in normal tibial growth plate and adult osteophytic human bone. (a) Morphologically, sections of the growth plate consist of areas of endochondral ossification with undifferentiated (small arrow head), proliferating (large arrow heads), mature (small arrow) and hypertrophic (large arrow) chondrocytes. Bar = 80 μ m. An inset of an area of the primary spongiosa is shown in b. (b) Numerous osteoblasts (small arrow heads) and multinucleated osteoclasts (large arrow heads) on the bone surface. Mononuclear cells within the bone marrow are also present (arrows). Bar = 60 μ m. (c) In the growth plate, AR is predominantly expressed by hypertrophic chondrocytes (large arrow heads). Minimal expression is observed in the mature chondrocytes (small arrow heads). The receptors are rarely observed in the proliferating chondrocytes (arrow). (d) In the primary spongiosa, the AR is predominantly and highly expressed by osteoblasts at modeling sites (arrow heads). Bar = 20 μ m. (e) In the osteophytes, AR is also observed at sites of endochondral ossification in undifferentiated (small arrow heads), proliferating (large arrow heads), mature (small arrows), and hypertrophic-like (large arrow) chondrocytes. Bar = 80 μ m. (f) A higher magnification of e) showing proliferating, mature, and hypertrophic-like chondrocytes (large arrows, small arrows, and very large arrows respectively) Bar = 40 μ m. (g) At sites of bone remodeling, the receptors are highly expressed in the osteoblasts (small arrow heads) and also in mononuclear cells in the bone marrow (large arrow heads). Bar = 40 μ m. (h) AR is not detected in osteoclasts (small arrow heads) Bar = 40 μ m. B, Bone; C, Cartilage; BM, Bone marrow (13).

prepubertal or senescent bone. Again, no differences were found between male and female samples, suggesting that differences in receptor number *per se* do not underlie development of a sexually dimorphic skeleton. Androgen and estrogen receptors have also been shown in bone marrow derived stromal cells (61), which are responsive to sex

steroids during the regulation of osteoclastogenesis. Because androgens are so important in bone development at the time of puberty, it is not surprising that ARs are also present in epiphyseal chondrocytes (13,62). Noble et al. (30) described AR expression mainly in the narrow zone of proliferating chondrocytes in the growth plate, with reduced expression in hypertrophied cells. The expression of ARs in such a wide variety of cell types known to be important for bone modeling during development, and remodeling in the adult, provides strong evidence for direct actions of androgens in bone and cartilage tissue. These results also presage the complexity of androgen effects on developing bone tissue.

Osteoclasts may be a target for sex steroid regulation as estrogen receptors have been reported to be present in osteoclastic cells (63), but a direct effect of androgens on osteoclast function has not been demonstrated. Mizuno et al. described the presence of AR immunoreactivity in mouse osteoclast-like multinuclear cells (64), but expression was not detected in *bona fide* osteoclasts in human bone slices (13). Because the major effects of androgens on skeletal remodeling and maintenance of bone mineral density seem to be mediated by cells of the osteoblast lineage (65), the biologic relevance of potential AR expression in osteoclasts is as yet unclear.

REGULATION OF ANDROGEN RECEPTOR EXPRESSION

The regulation of AR expression in osteoblasts is incompletely characterized. Homologous regulation of AR by androgen has been described that is tissue specific; upregulation by androgen exposure is seen in a variety of osteoblastic cells (20,23,66,67) whereas in prostatic tissue, downregulation of AR after androgen exposure is observed. The androgen mediated upregulation of AR observed in osteoblasts, at least in part, occurs through changes in AR gene transcription (Fig. 4). As in other tissues, increased AR protein stability may also play a part. No effect, or even inhibition, of AR mRNA by androgen exposure in other osteoblastic models has also been described (24,68). The mechanism(s) that underlies tissue specificity in autologous AR regulation, and the possible biological significance of distinct autologous regulation of AR, is not yet understood. It is possible that receptor upregulation by androgen in bone may result in an enhancement of androgen responsiveness at times when androgen levels are rising or elevated. In addition, AR expression in osteoblasts may be upregulated by exposure to glucocorticoids, estrogen or 1,25-dihydroxyvitamin D₃ (24). Except for the immunocytochemical detection of AR expression in bone slices described previously, regulation during osteoblast differentiation has not been well characterized. A preliminary report describes changes in AR mRNA abundance during osteoblast in vitro differentiation (Wiren, submitted #154). Whether any other hormones, growth factors or agents influence AR expression in bone is unknown.

EFFECTS OF ANDROGENS ON THE PROLIFERATION AND APOPTOSIS OF OSTEOBLASTIC CELLS

Androgens have direct effects on osteoblast proliferation and expression in vitro. The effect of androgen exposure on osteoblast proliferation remains controversial; both stimulation and inhibition of osteoblast proliferation have been reported as summarized in Table 1. Benz et al. (18) have shown that prolonged androgen exposure in the presence of serum inhibited proliferation (cell counts) by 15–25% in a transformed human osteoblastic line

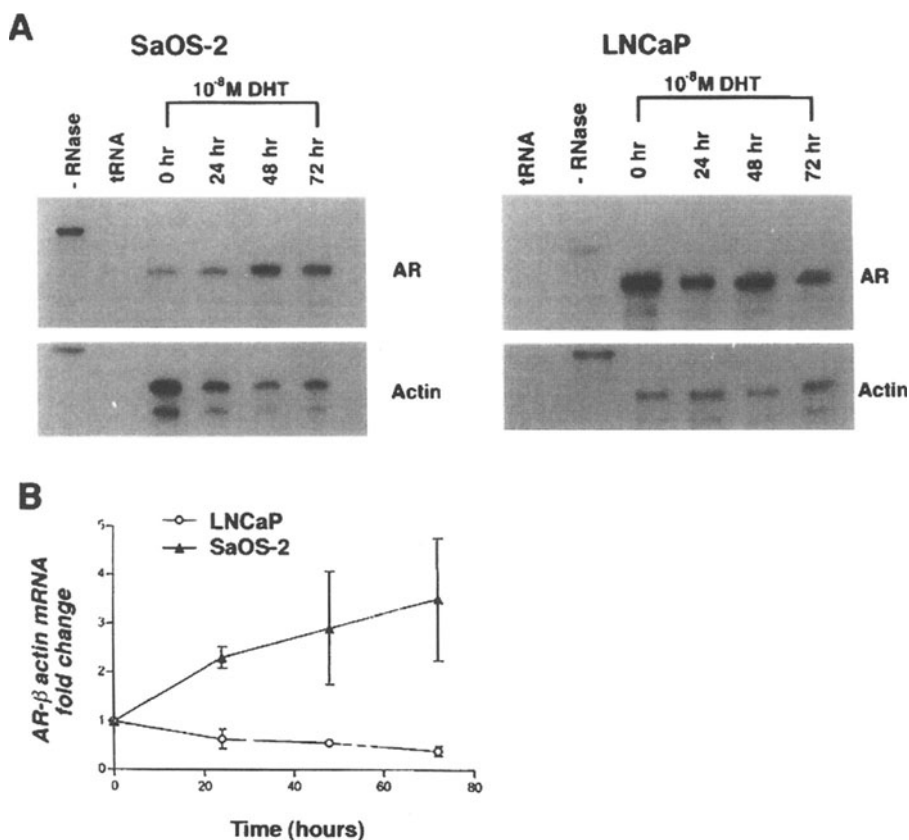


Fig. 4. Dichotomous regulation of AR mRNA levels in osteoblast-like and prostatic carcinoma cell lines after exposure to androgen. **(A)** Time course of changes in AR mRNA abundance after DHT exposure in human SaOS-2 osteoblastic cells and human LNCaP prostatic carcinoma cells. To determine the effect of androgen exposure on hAR mRNA abundance, confluent cultures of either osteoblast-like cells (SaOS-2) or prostatic carcinoma cells (LNCaP) were treated with 10^{-8} M DHT for 0, 24, 48, or 72 h. Total RNA was then isolated and subjected to RNase protection analysis with 50 μ g total cellular RNA from SaOS-2 osteoblastic cells and 10 μ g total RNA from LNCaP cultures. **(B)** Densitometric analysis of AR mRNA steady state levels. The AR mRNA to β -actin ratio is expressed as the mean \pm SE compared to the control value from three to five independent assessments (66).

(TE-85). Testosterone and DHT were nearly equally effective regulators. Hofbauer et al. (69) examined the effect of DHT exposure on proliferation in hFOB/AR-6, an immortalized human osteoblastic cell line stably transfected with an AR expression construct (with ~ 4000 receptors/cell). In this line, DHT treatment inhibited cell proliferation by 20–35%. Finally, Kasperk et al. (24) reported that prolonged DHT pretreatment inhibited normal human osteoblastic cell proliferation (cell counts) in cultures pretreated with DHT.

In contrast, the same group (70,71) also demonstrated in osteoblast-like cells in primary culture (murine, passaged human) that a variety of androgens increase DNA synthesis ($[^3\text{H}]$ thymidine incorporation) up to nearly 300%. Again, testosterone and nonaromatizable androgens (DHT and fluoxymesterone) were nearly equally effective regulators. Consistent with increased proliferation, testosterone and DHT have also been

Table 1
Complex Effects of Androgens on Proliferation of Osteoblastic Cells

<i>Cells</i>	<i>Steroid</i>	<i>Conditions</i>	<i>Change</i>	<i>Ref.</i>
h TE-85 (osteosarcoma)	DHT (10 nM), 72 h	2% FBS	↓ 25%	18
	T (10 nM), 72 h	2% FBS	↓ 20%	18
h FOB/AR6 (immortalized)	DHT (10 nM), 6 d	1% csFBS	↓ 30%	69
m MC3T3-E1 (immortalized)	DHT (10 nM), 24 h	1.5% csFBS	↑ ~32%	22
	T (10 nM), 24 h	1.5% csFBS	↑ ~28%	22
m MC3T3-E1 (immortalized)	DHT (10 nM), 72 h	SF	↑ ~15%	27
r normal calvarial OBs	DHT (10 nM), 5 d	1 st psg, 5% csFBS	↑ ~48%	87
r normal long bone OBs	T (50 nM), 5 d	1 st psg, 5% csFBS	↑ ~60%	87
r normal long bone OBs	DHT (10 nM), 24 h	1 st psg, 2% csFBS	↑ ~180%	114
	T (100 nM), 24 h	1 st psg, 2% csFBS	↑ ~100%	114
r normal explanted OBs	DHT (10 nM), 8 d	1 st psg, 5% csFBS	↑ ~150%	87
h mandibular OBs	DHT (1 nM), 3 d	1 st /2 nd psg, SF	↑ ~46%	24
h iliac crest OBs	DHT (1 nM), 3 d	1 st /2 nd psg, SF	no effect	24
h cortical OBs	DHT (10 nM), 48 h	1 st /2 nd psg, 1% csFBS	↑ ~230%	50
h cortical OBs	DHEA (10 nM), 48 h	1 st /2 nd psg, 1% csFBS	↑ ~170	50
h normal OBs	DHT (1 nM), 48 h; (24 h pretreatment 10 nM DHT)	1 st /2 nd psg, SF	↓ ~40%	24
r diaphysis	DHT (50 µg), 24 h	20 d rats; in vivo	↑ 98%	72
r epiphysis	DHT (50 µg), 24 h	20 d rats; in vivo	↑ 83%	72

h, human; m, mouse; r, rat; OBs, osteoblasts; T, testosterone; cs, charcoal-stripped; SF, serum-free; psg, passage.

reported to cause an increase in creatine kinase activity and [³H]thymidine incorporation into DNA in rat diaphyseal bone (72). Variable results have also been reported with the adrenal androgen DHEA on osteoblast proliferation; DHEA was shown to stimulate osteoblast proliferation, but with less potency than DHT (50), however, no effect of DHEA alone has also been described (73). The differences observed with androgen-mediated changes in osteoblastic cell proliferation may be due to the variety of model systems employed (transformed osteoblastic cells vs passaged normal cells from different species) and/or may reflect differences in the culture conditions (e.g., state of differentiation, receptor number, times of treatment, phenol red-containing vs phenol red-free, or serum-containing, charcoal-stripped vs serum-free). These differences suggest an underlying biologic complexity for androgen regulation of osteoblast proliferation.

As a component of control of osteoblast survival, it is also important to consider programmed cell death, or apoptosis (74). A variety of skeletal cell types have been shown to undergo apoptosis (75,76). In particular, as the osteoblast population differentiates in vitro, the mature bone cell phenotype undergoes apoptosis (77). Modulation of bone cell apoptosis by steroid hormones has been shown: glucocorticoids enhance apoptosis of osteoclasts (78) and osteoblasts/osteocytes (79,80), which estrogen treatment prevents (81). Further-

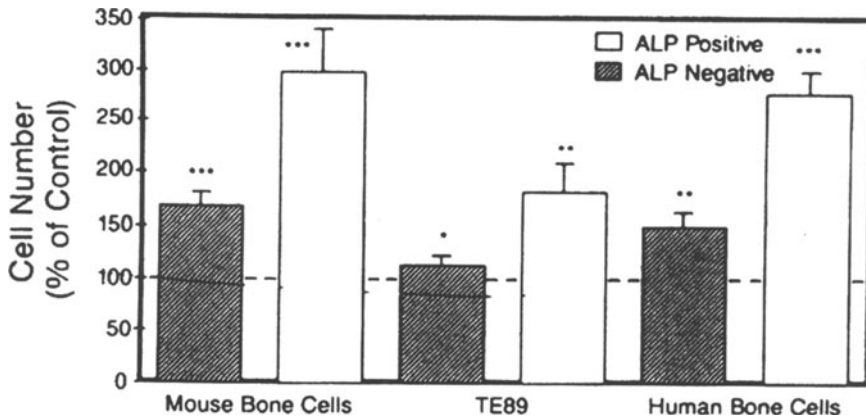


Fig. 5. Effect of DHT on ALP positive (ALP⁺) and ALP negative (ALP⁻) cells in normal mouse, normal human osteoblast-line and human osteosarcoma (TE89) monolayer cell culture. (***) = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.1$. control values in cells per mm² for mouse bone cells were: 90 ± 5 ; TE89 cells: 75 ± 7 ; human bone cells: 83 ± 14 (70).

more, evidence shows that the osteocytic population is particularly sensitive to the effects of estrogen withdrawal, which induces apoptosis (82,83). Androgen exposure has been shown to influence apoptosis in other tissues (84,85), but the effects of either androgen exposure or androgen withdrawal in bone have not been described.

EFFECTS OF ANDROGENS ON THE DIFFERENTIATION OF OSTEOBLASTIC CELLS

Osteoblast differentiation is often characterized by changes in alkaline phosphatase activity and/or alterations in the expression of important extracellular matrix proteins, such as type I collagen, osteocalcin, and osteonectin. Enhanced osteoblast differentiation, as measured by increased matrix production, has been shown to result from androgen exposure. Androgen treatment in both normal osteoblasts and transformed clonal human osteoblastic cells (TE-89) appears to increase the proportion of cells expressing alkaline phosphatase activity, thus representing a shift toward a more differentiated phenotype (Fig. 5) (70). Kasperk et al. subsequently reported dose-dependent increases in alkaline phosphatase activity in both high and low-alkaline phosphatase subclones of SaOS2 cells (86) and human osteoblastic cells (50). However, there are also reports in a variety of model systems of androgens either inhibiting (69) or having no effect on alkaline phosphatase activity (23,87), which may reflect both the complexity and dynamics of osteoblastic differentiation. There are also reports of androgen-mediated increases in type I α -1 collagen protein and mRNA levels (18,86–88) and increased osteocalcin mRNA or protein secretion (50,88). Consistent with increased collagen production, androgen treatment has also been shown to stimulate mineral accumulation in a time- and dose-dependent manner (23,50,89). These results suggest that under certain conditions androgens can enhance osteoblast differentiation and may thus play an important role in the regulation of bone matrix production and/or organization. This effect is also consistent with an overall stimulation of bone formation, as is observed in vivo after androgen treatment.

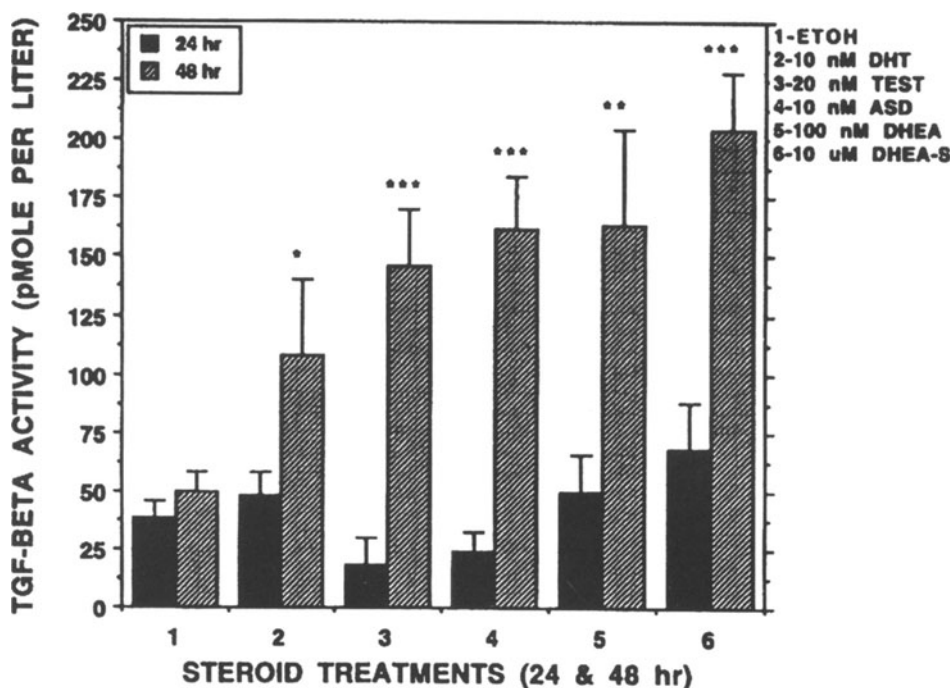


Fig. 6. Induction of total TGF- β activity by gonadal and adrenal androgens in human osteoblast (hOB) cell conditioned-media. The cells were treated for 24–48 h with vehicle or steroids. After treatment, the conditioned-media was saved and processed for the TGF- β bioassay. The results are presented as the mean \pm SEM of 3–4 experiments; * P < 0.05; ** P < 0.02, *** P < 0.0005 (Behren's-Fisher t -test) compared to the 48 h ethanol control. ETOH, ethanol; TEST, testosterone; DHT, dihydrotestosterone; ASD, androstenedione; DHEA, dehydroepiandrosterone; DHEA-S, DHEA-sulfate (51).

INTERACTION WITH OTHER FACTORS TO MODULATE BONE FORMATION AND RESORPTION

The effects of androgens on osteoblast activity must certainly also be considered in the context of the very complex endocrine, paracrine, and autocrine milieu in the bone microenvironment. Systemic and/or local factors can act in concert, or antagonize, to influence bone cell function. This has been well described with regard to modulation of the effects of estrogen on bone (see for example Horowitz, 1993 #127; Kassem, 1996 #60; Kawaguchi, 1995 #128). Androgens have also been shown to regulate well-known modulators of osteoblast proliferation or function. The most extensively characterized growth factor influenced by androgen exposure is TGF- β . TGF- β is stored in bone matrix (the largest reservoir for TGF- β in the body) in a latent form and has been shown under certain conditions to be either a mitogen for osteoblasts (90,91), or to inhibit proliferation (92–94). Androgen treatment has been shown to increase TGF- β activity in human osteoblast primary cultures (Fig. 6). The expression of some TGF- β mRNA transcripts (apparently TGF- β 2) was increased, but no effect on TGF- β 1 mRNA abundance was observed (51,71). At the protein level, specific immunoprecipitation analysis

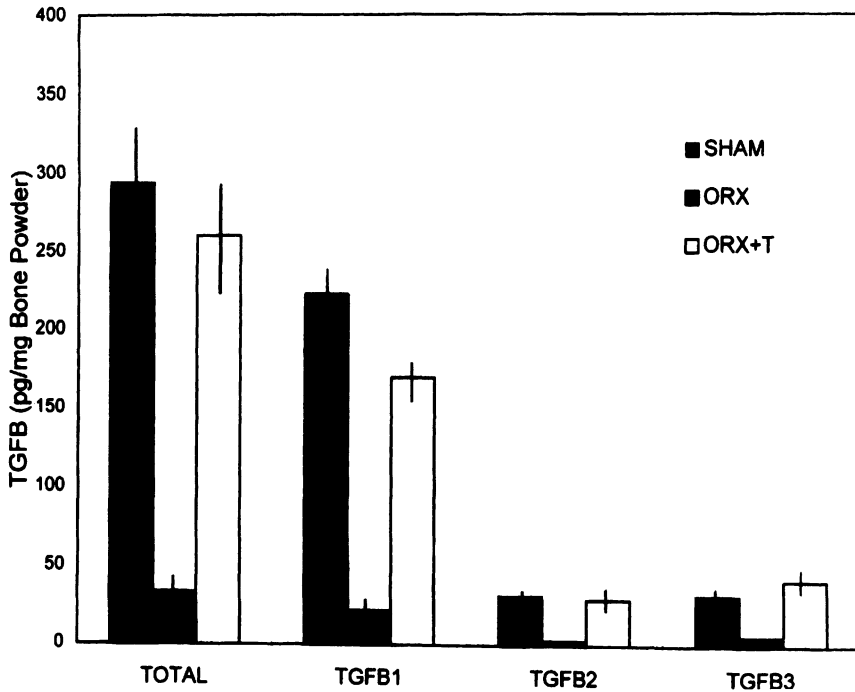


Fig. 7. Effects of orchiectomy and testosterone replacement on isoforms of TGF β in long bones. Results are mean \pm SE of 4–6 animals. Rats underwent sham operation or orchiectomy and one week later were given either placebo or 100 mg of testosterone in 60-d slow release pellets. Specimens were obtained 6 wk after surgery. All forms of TGF β were reduced by orchiectomy (at least $p < 0.0002$), while there was no change in those with testosterone replacement (96).

reveals DHT-mediated increases in TGF- β activity to be predominantly TGF- β 2 (51,71). DHT has also been shown to inhibit both TGF- β gene expression and TGF- β -induced early gene expression, effects that correlate with growth inhibition observed in this cell line (69). The TGF- β -induced early gene has been shown to be a transcription factor that may mediate some TGF- β effects (95). However, TGF- β 1 mRNA levels are increased by androgen treatment in human clonal osteoblastic cells (TE-89), under conditions where osteoblast proliferation is slowed (18). These results are consistent with the notion that TGF- β may mediate the complex androgen effects on osteoblast proliferation. Furthermore, the specific TGF- β isoform may determine osteoblast responses. It is interesting to note that at the level of bone, orchiectomy drastically reduces bone content of TGF- β levels, and testosterone replacement prevents its occurrence (96) (Fig. 7). These data support the findings that androgens influence cellular expression of TGF- β and suggest that the bone loss associated with castration is related to or associated with a reduction in growth factor abundance induced by androgen deficiency.

Other growth factor systems may also be influenced by androgens. Conditioned media from DHT treated normal osteoblast cultures are mitogenic, and DHT pretreatment increases the mitogenic response to fibroblast growth factor and to insulin-like growth factor II (IGF-II) (71). In part, this may be due to slight increases in IGF-II binding in DHT-treated cells (71), as IGF-I and IGF-II levels in osteoblast-conditioned media are not affected by androgen (71,97). Although most studies have not found regulation of IGF-I or IGF-II abundance by androgen exposure (22,71,97), it has been shown that IGF-I

mRNA levels are significantly upregulated by DHT (98). Androgens may also modulate the expression of components of the AP-1 transcription factor, as has been shown with androgen inhibition of *c-fos* expression in proliferating normal osteoblast cultures (51). Thus, androgens may accelerate osteoblast differentiation via a mechanism whereby growth factors or other mediators of differentiation are regulated by androgen exposure.

Finally, androgens may modulate responses to other important osteotropic hormones/regulators. Testosterone and DHT specifically inhibit the cAMP response elicited by parathyroid hormone or parathyroid hormone-related protein in the human clonal osteoblast-like cell line SaOS-2, whereas the inactive or weakly active androgen 17 α -epitestosterone had no effect (Fig. 8). This inhibition may be mediated via an effect on the parathyroid hormone receptor-G_s-adenylyl cyclase (99–101). The production of prostaglandin E₂ (PGE₂), another important regulator of bone metabolism, is also affected by androgens. Pilbeam and Raisz (102) showed that androgens (either DHT or testosterone) were potent inhibitors of both parathyroid hormone (Fig. 9) and interleukin-1-stimulated PGE₂ production in cultured neonatal mouse calvaria. The effects of androgens on parathyroid hormone action and PGE₂ production suggest that androgens could act to modulate (i.e., reduce) bone turnover in response to these agents.

Finally, both androgen (14) and estrogen (103,104) inhibit the production of interleukin-6 by osteoblastic cells (see ref. 105). In stromal cells of the bone marrow, androgens have been shown to have potent inhibitory effects on the production of interleukin-6 (Table 2) and the subsequent stimulation of osteoclastogenesis by marrow osteoclast precursors (61). Interestingly, adrenal androgens (androstenediol, androstenedione, dehydroepiandrosterone) have similar inhibitory activities on interleukin-6 gene expression and protein production by stroma (61). The loss of inhibition of interleukin-6 production by androgen may contribute to the marked increase in bone remodeling and resorption that has been shown to follow orchidectomy. Moreover, androgens inhibit the expression of the genes encoding the two subunits of the IL-6 receptor (gp80 and gp130) in the murine bone marrow, another mechanism that may blunt the effects of this osteoclastogenic cytokine in intact animals (106). In this aspect, the effects of androgens seem to be very similar to those of estrogen, which may also inhibit osteoclastogenesis via mechanisms that involve interleukin-6 inhibition.

METABOLISM OF ANDROGENS IN BONE-AROMATASE AND 5 α -REDUCTASE ACTIVITIES

There is abundant evidence in a variety of tissues that the eventual cellular effects of androgens may be the result not only of direct action of androgen, but also of the effects of sex steroid metabolites formed as the result of local enzyme activities. Probably the most significant of these androgen metabolites are estradiol (formed by the aromatization of testosterone) and 5 α -DHT (the result of 5 α reduction of testosterone). Evidence shows that both aromatase and 5 α -reductase activities are present in bone tissue, at least to some measurable extent, but the biologic relevance is still controversial.

5 α -reductase activity was first described in crushed rat mandibular bone by Vittek et al. (107); and Schweikert et al. (108) reported similar findings in crushed human spongiosa. Two different 5 α -reductase genes encode type 1 and type 2 isozymes in many mammalian species (109); type 1 is expressed in human and rat osteoblastic osteosarcoma cell lines (110) but the isozyme present in human bone has been incompletely

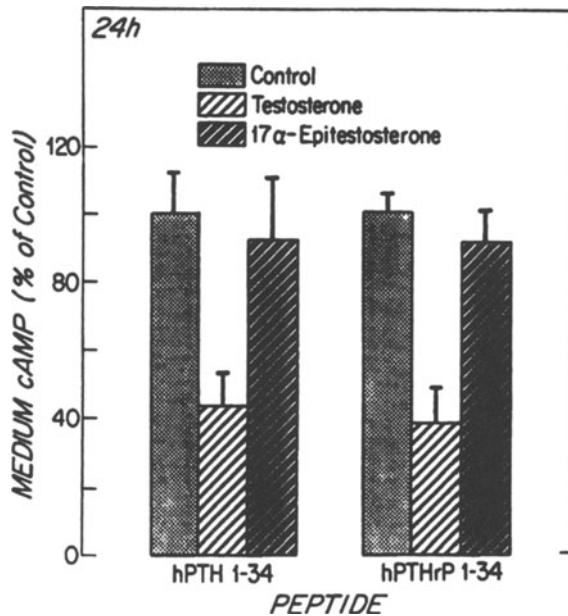


Fig. 8. Actions of testosterone and 17 α -epitestosterone on cAMP accumulation stimulated by hPTH¹⁻³⁴ (5.0 nM) or hPTHrP¹⁻³⁴ (5.0 nM) in human SaOS-2 cells. Cells were pretreated without or with the steroid hormones (10^{-9} M) for 24 h. Each bar gives the mean value, and the brackets give the SE for 4–5 dishes (99).

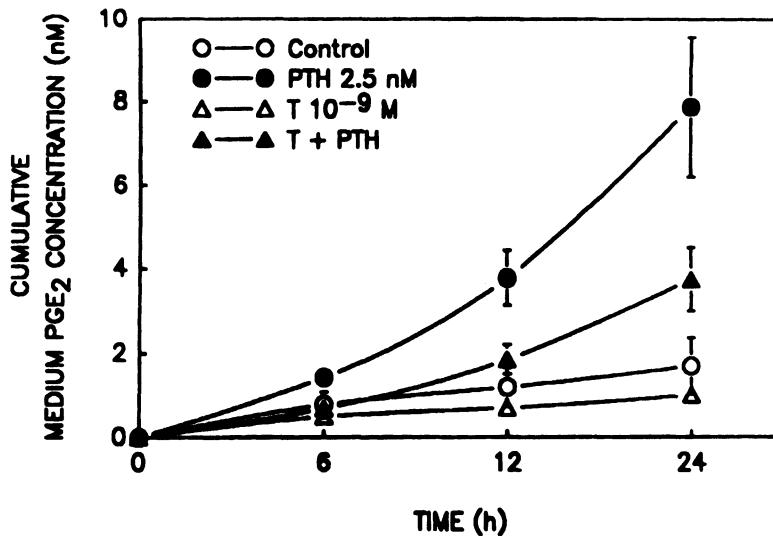


Fig. 9. Effect of testosterone (T) on PTH-stimulated PGE₂ production in cultured neonatal calvariae as a function of time. Each bone was precultured for 24 h in 1 mL medium with or without 10^{-9} M T and then transferred to similar medium with 2.5 nM PTH. Media were sampled (0.1 mL) at the indicated times. Data were corrected for the media removed. Each point represents the mean \pm SEM for six bones in one experiment. The effect of T on PTH-stimulated PGE₂ production was significant ($P < 0.05$) at 6, 12, and 24 h (102).

Table 2
Effect of Androgens on Cytokine-Induced IL-6 Production
by Murine Bone Marrow Stromal Cells

<i>Treatment</i>	<i>IL-6 (ng/mL per 10⁵ cells)</i>
IL-1 + TNF	4.27 ± 1.43
IL-1 + TNF+ testosterone (10 ⁻¹² M)	3.87 ± 0.33
IL-1 + TNF+ testosterone (10 ⁻¹¹ M)	2.90 ± 0.42
IL-1 + TNF+ testosterone (10 ⁻¹⁰ M)	2.09 ± 0.33
IL-1 + TNF+ testosterone (10 ⁻⁹ M)	1.12 ± 0.49
IL-1 + TNF+ testosterone (10 ⁻⁸ M)	1.03 ± 0.04
IL-1 + TNF+ testosterone (10 ⁻⁷ M)	1.01 ± 0.48
IL-1 + TNF+ dihydrotestosterone (10 ⁻¹² M)	4.05 ± 0.19
IL-1 + TNF+ dihydrotestosterone (10 ⁻¹¹ M)	2.97 ± 0.48
IL-1 + TNF+ dihydrotestosterone (10 ⁻¹⁰ M)	2.31 ± 0.86
IL-1 + TNF+ dihydrotestosterone (10 ⁻⁹ M)	1.72 ± 0.43
IL-1 + TNF+ dihydrotestosterone (10 ⁻⁸ M)	0.65 ± 0.21
IL-1 + TNF+ dihydrotestosterone (10 ⁻⁷ M)	1.41 ± 0.82

Murine stromal cells (+/+LDA11 cells) were cultured for 20 h in the absence or the presence of different concentrations of either testosterone or dihydrotestosterone. Then IL-1 (500 U/mL) and TNF (500 U/mL) were added and cells were maintained for another 24 h in culture. Values indicate means (± SD) of triplicate cultures from one experiment. Data were analyzed by one-way ANOVA. **P* < 0.05 vs cells not treated with steroids as determined by Dunnett's test. Neither testosterone nor dihydrotestosterone had an affect on cell number (61).

characterized. In osteoblast-like cultures derived from orthopedic surgical waste, androstenedione (the major circulating androgen in women) can be reversibly converted to testosterone via 17 β -hydroxysteroid dehydrogenase activity and to 5 α -androstanedione via 5 α -reductase activity, whereas testosterone is converted to DHT via 5 α -reductase activity (111). The principal metabolite of androstenedione is α -androstanedione in the 5 α -reductase pathway, and testosterone in the 17 β -hydroxysteroid dehydrogenase pathway. Essentially the same results were reported in experiments with human epiphyseal cartilage and chondrocytes (112). In general, the *K_m* values for bone 5 α -reductase activity are similar to those in other androgen responsive tissues (22,108).

The cellular populations in these studies were mixed and hence the specific cell type responsible for the activity is unknown. Interestingly, Turner et al. found that periosteal cells do not have detectable 5 α -reductase activity (113), raising the possibilities that the enzyme may be functional in only selected skeletal compartments, and that testosterone may be the active metabolite at this clinically important site (see also ref. 114).

From a clinical perspective, the general importance of this enzymatic pathway is suggested by the presence of skeletal abnormalities in patients with 5 α -reductase type 2 deficiency (115). However, Bruch et al. (111) found no significant correlation between enzyme activities and bone volume. In mutant null mice lacking 5 α -reductase type 1 (mice express very little type 2 isozyme), the effect on the skeleton cannot be analyzed due to midgestational fetal death (116). Treatment of male animals with finasteride (an inhibitor of type 2 5 α -reductase activity) does not recapitulate the effects of castration (117), indicating that reduction of testosterone to DHT by the type 2 isozyme is not a

major determinant in the effects of gonadal hormones on bone. While available data point to a possible role for 5α -reduction in the mechanism of action for androgen in bone, the clinical impact of this enzyme, the isozyme which may be involved, whether it is uniformly present in all cells participating in bone modeling/remodeling, or whether local activity is important at all remains uncertain.

The biosynthesis of estrogens from androgen precursors is catalyzed by the microsomal enzyme aromatase cytochrome P450 (P450_{arom}, the product of the CYP19 gene). It is an enzyme known to be both expressed and regulated in a very pronounced tissue-specific manner (118). Aromatase activity has been reported in bone from mixed cell populations derived from both sexes (119–121) and from osteoblastic cell lines (22, 122–124). Aromatase expression in intact bone has been documented by *in situ* hybridization and immunohistochemical analysis (121) (Table 3). Aromatase mRNA is expressed predominantly in lining cells, chondrocytes, and some adipocytes, but there is no detectable expression in osteoclasts. At least in vertebral bone, the aromatase fibroblast (1b type) promoter is predominantly utilized (121). The enzyme kinetics in bone cells seem to be similar to those in other tissues, although the V_{\max} may be increased by glucocorticoids (123).

Aromatase can produce the potent estrogen estradiol, but also can produce in the weaker estrogen estrone from its adrenal precursors androstenedione and dehydroepiandrosterone (119). In addition to aromatase itself, osteoblasts also express 17β -hydroxysteroid dehydrogenase (110, 125) that is able to interconvert estradiol and estrone and also to convert androstenedione to testosterone; and estrone sulfatase to hydrolyze estrone sulfate to estrone (122). Nawata et al. (119) have reported that dexamethasone and $1\alpha,25(\text{OH})_2\text{D}_3$ synergistically enhance aromatase activity and aromatase mRNA (P450_{arom}) expression in human osteoblast-like cells. There is no other information concerning the regulation of aromatase in bone, although this is an area of obvious interest given the potential importance of the enzyme and its regulation by a variety of mechanisms (including androgens and estrogens) in other tissues (118, 126).

The clinical impact of aromatase activity has been suggested by the reports of skeletal changes in women (127) and men (128, 129) with aromatase deficiencies. The presentation of men with aromatase deficiency is very similar to that of a man with estrogen receptor- α deficiency, namely an obvious delay in bone age, lack of epiphyseal closure, and tall stature (130), suggesting that aromatase (and thus estrogen action) has a substantial role to play during skeletal development in the male. In one case, estrogen therapy of a man with an aromatase deficiency was associated with an increase in bone mass (131). Inhibition of aromatization in young growing orchidectomized males, with the nonsteroidal inhibitor vorozole, results in decreases in bone mineral density and changes in skeletal modeling, as does castration which reduces both androgens and estrogens. However, vorozole therapy induces less dramatic effects on bone turnover (132). Inhibition of aromatization in older orchidectomized males resembles castration with similar increases in bone resorption and bone loss, suggesting that aromatase activity may also play a role in skeletal maintenance in males (133). These studies herald the importance of aromatase activity (and estrogen) in the mediation of androgen action in bone.

The finding of these enzymes in bone clearly raises the difficult issue of the origin of androgenic effects. Do they arise from direct androgen effects (as is suggested by the actions of nonaromatizable androgens) or to some extent from the local production of estrogenic intermediates or both? Even so, there is substantial evidence that some, if in

Table 3
Steroid Metabolism in Human Bone

Crystallization	Solvent	Dihydrotestosterone		Androstenedione	
		³ H cpm/mg	¹⁴ C cpm/mg	³ H cpm/mg	³ H/ ¹⁴ C
1	Acetone	409	26	811	14
2	Benzene-heptane	408	25	811	14
3	Ethylacetate-cyclohexane	411	26	786	13
4	Ethylether-hexane	411	28	775	13
5	Methanol	393	26	791	16
Mother liquor		405	24	848	16

See ref. 108.

Confirmation by recrystallization of the identities of [³H]dihydrotestosterone and [³H]androstenedione recovered following the incubation of normal and osteoporotic human bone (ground spongiosa) with [1,2,7,7-³H]testosterone. Pooled samples from 18 separate incubations of bone from various anatomical origins were chromatographed by preparative thin-layer chromatography. Material tentatively identified as [³H]dihydrotestosterone and [³H]androstenedione, respectively was mixed with 200 mg of the appropriate carrier steroid and with ¹⁴C labeled steroid for recrystallization as described in the text.

fact not most, of the biologic actions of androgens in the skeleton are direct. As noted previously, both in vivo and in vitro systems reveal the effects of the nonaromatizable androgen DHT to be essentially the same as those of testosterone (*vida infra*). In addition, blockade of the AR with the receptor antagonist flutamide results in osteopenia as a result of reduced bone formation (7). These reports clearly indicate that androgens, independent of estrogenic metabolites, have primary effects on osteoblast function. However, the clinical reports of subjects with aromatase deficiency also highlight the relevance of androgen metabolism to bio-potent estrogens in bone. The elucidation of the regulation of skeletal steroid metabolism, and the potential mechanisms by which androgenic and estrogenic effects are coordinated, will have important physiological, pathophysiological, and therapeutic implications.

DIRECT EFFECTS OF ANDROGENS ON OTHER CELL TYPES IN BONE IN VITRO

Similar to the effects noted in osteoblastic populations, androgens regulate chondrocyte proliferation and expression. Androgen exposure promotes chondrogenesis as shown with increased creatine kinase and DNA synthesis after androgen exposure in cultured epiphyseal chondrocytes (62,134). Increased [³⁵S] sulfate incorporation into newly synthesized proteoglycan (135) and increased alkaline phosphatase activity (136) are androgen mediated. Regulation of these effects is obviously complex, as they were dependent on the age of the animals and the site from which chondrocytes were derived. Thus, in addition to effects on osteoblasts, multiple cell types in the skeletal milieu are regulated by androgen exposure.

GENDER-SPECIFICITY IN THE ACTIONS OF SEX STEROIDS

Although controversial, there may be gender specific responses in osteoblastic cells to sex steroids. Somjen and colleagues (137,138) have shown that the increase in creatine kinase that occurs from bone cells in vivo and in vitro is gender specific (i.e., male animals, or cells derived from male bones, respond only to androgens, whereas females or female-derived cells respond only to estrogens). This gender specificity appears to depend on the previous history of exposure of animals to androgens (or estrogens). How much gender-specific effects might affect bone metabolism in the intact animal is completely unknown.

In addition, in most mammals, there is a marked gender difference in morphology that results in a sexually dimorphic skeleton. The mechanisms responsible for these differences are obviously complex and presumably involve both androgenic and estrogenic actions on the skeleton. It is becoming increasingly clear that estrogens are particularly important for the regulation of epiphyseal function and act to reduce the rate of longitudinal growth via influences on chondrocyte proliferation and function, as well as on the timing of epiphyseal closure (5). Androgens, on the other hand, appear to have opposite effects on skeletal development. Androgens tend to promote long bone growth, chondrocyte maturation, and metaphyseal ossification. Furthermore, the most dramatic effect of androgens is on bone size, in particular cortical thickness (5,139) as androgens appear to have gender-specific effects on periosteal bone formation (6). This difference of course has biomechanical implications, since thicker bones are stronger bones. At the same time, the response of the adult skeleton to the same intervention results is distinct

responses in males and females. For example, in a model of disuse osteopenia, antiorthostatic suspension results in significant reduction in bone formation rate at the endocortical perimeter in males. In females however, a decrease in bone formation rate occurred along the periosteal perimeter (140). Gender-specific responses in vivo and in vitro, and the mechanism(s) that underlie such responses in bone cells, may thus have significant implications in treatment options for metabolic bone disease.

SUMMARY

Thus, the effects of androgens on bone health are both complex and pervasive. The effects of androgens are particularly dramatic during growth in boys, but almost certainly play an important role during this period in girls as well. Throughout the rest of life, androgens affect skeletal function and maintenance in both sexes. Nevertheless, given this importance, relatively little has been done to unravel the mechanisms by which androgens influence the physiology and pathophysiology of bone, and there is still much to be learned about the roles of androgens at all levels. The interaction of androgens and estrogens, and how their respective actions can be utilized for specific diagnostic and therapeutic benefit, are important but unanswered issues. With an increase in the understanding of the nature of androgen effects will come greater opportunities to use their positive actions in the prevention and treatment of a wide variety of bone disorders.

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18

Androgens and Skeletal Homeostasis

Potential Clinical Implications of Animal Data

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INTRODUCTION

Males deprived of androgen action, due to a lack of functional androgen receptors, have a female and not male phenotype (1,2), indicating that the development of the male phenotype depends on androgen action. The skeletal male phenotype is characterized by stronger bones than in females, primarily because male bones have greater size and not because they contain more mineral for similar size (3,4). It would seem logical, therefore, to assume that androgen action is particularly important for gender differences in bone size. Theoretically, androgen action may affect male skeletal homeostasis in utero, during the neonatal period, as well as before and after puberty. Females, on the other hand, do not secrete sex steroids before early puberty. Therefore, it is unlikely that sex steroids affect skeletal homeostasis before early puberty in the female.

Indirect androgen action outside bone tissue, however, may be the main determinant of early gender differences in bone size. Indeed, sex differences in skeletal growth also depend on a gender-specific growth hormone—IGF-I secretion in the pituitary, programmed by neonatal androgen secretion (5,6).

To a large extent, skeletal growth is associated with overall body growth and growth-related changes in body composition (7). In this respect, the strong relationship between lean body mass and bone mineral suggests that skeletal homeostasis is associated with

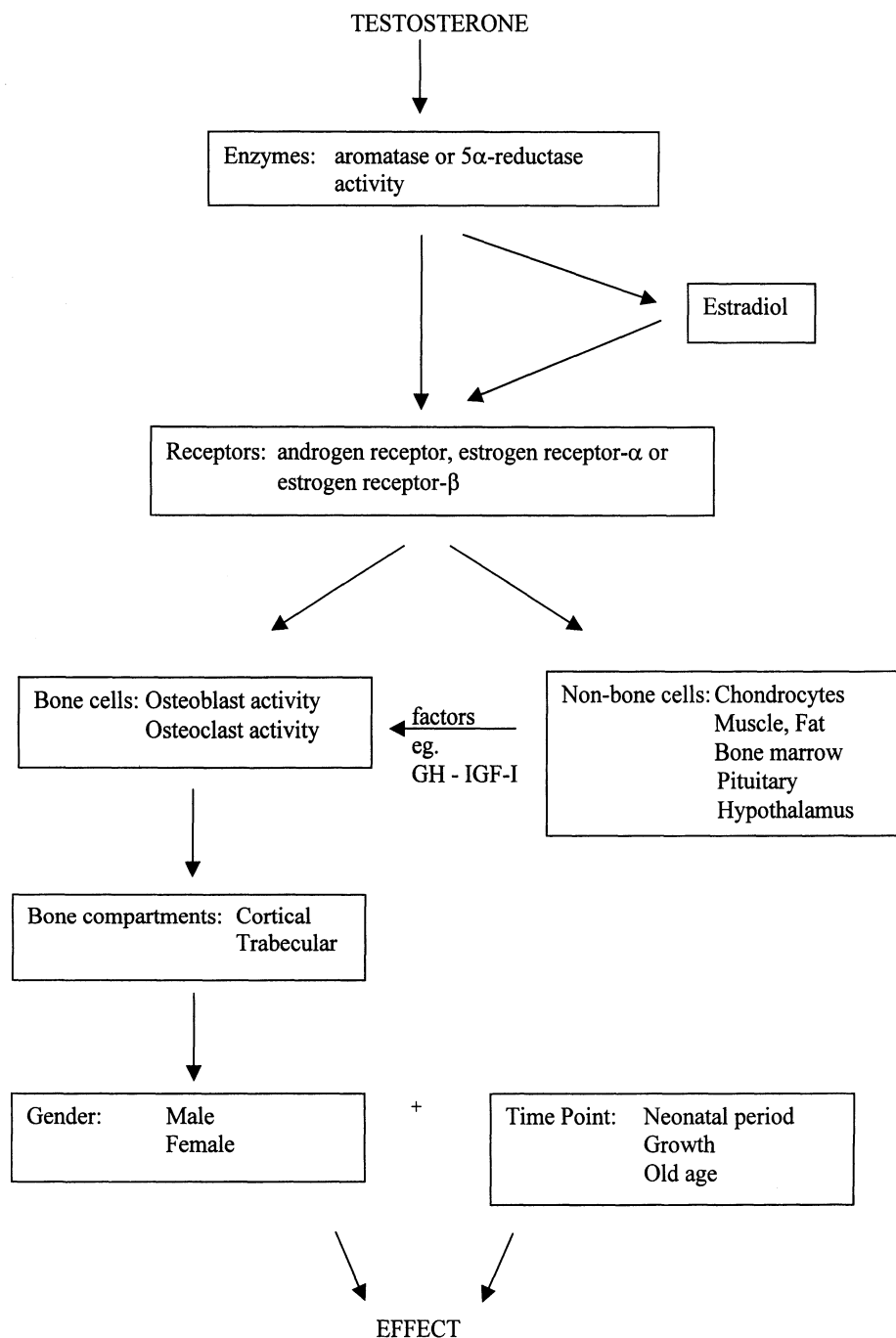


Fig. 1. This figure illustrates the complexity of skeletal androgen action in an animal model. According to this “mathematical model,” the end-result of testosterone action may depend on metabolism (androgen versus estrogen) and the presence of receptors in bone cells (in different compartments) and non-bone cells. Finally, the effects may ultimately differ according to gender and timing (neonatal period, growth and old age).

changes in mechanical strain induced by muscle, which is reflected by the measurement of lean body mass (7). In line with this theory (8), sex steroids only change mechanical

setpoints of skeletal homeostasis. Therefore, skeletal actions of sex steroids are always to be discussed in light of their possible indirect effects on body composition. For instance, evolution has particularly taken care of the female, probably because she needs more bone mineral for reproduction. Therefore, bone mineral increases more than lean body mass during puberty in women than men (7); an advantage they lose after menopause. Overall, men gain more bone mineral because they also gain more lean body mass. However, men do not have more bone mineral with respect to volume (4). Moreover, men have less bone with respect to lean body mass than women (at least during female reproductive ages).

By 1969, Saville had already made similar fundamental observations in rats (9). Relative to body weight, female rats have more bone mineral, an advantage (in accordance with women) that they lose after ovariectomy. Therefore, extra estrogen in females (compared to males) is probably associated with the relative extra bone mineral that females acquire during puberty. In many ways, it would seem, the adaptation of the skeleton to both mechanical force and reproduction is similar in rodents and in humans. In this regard, the rodent seems the appropriate model for the study of the regulation of these processes by sex steroids. One of the important questions in this respect is: what is the specific skeletal role of androgens? Do androgens, like estrogens, play a role in skeletal homeostasis during and after puberty? The complexity of this question is illustrated by Fig. 1. The effects of androgens, such as testosterone, may indeed depend on many variables, such as their action as pro-hormones for estrogens. We will mainly focus on animal data with respect to skeletal androgen action. However, we prefer to do this in light of their possible relevance for humans. Therefore, references to and comparisons with human data are indicated throughout the chapter.

ANDROGEN ACTION IN ANIMAL MODEL SYSTEMS

The best characterized model for the study of androgen action is the orchidectomized male rat (and, to a lesser extent, mouse). Other orchidectomized animal models such as dogs (10), baboons (11), and guinea pigs (12) are much less documented.

Androgen deficiency of the male rodent, as induced by orchidectomy, has well-established effects on its skeleton. In this chapter, we will review some of these changes in both rats and mice, with special emphasis on the possible relevance for humans.

Effects of Orchidectomy in the Male Rat

POSSIBLE LIMITATIONS OF THE ORCHIDECTOMIZED MODEL

Important extraskeletal side-effects of orchidectomy in rats include decreases in body weight gain, food intake, growth rate and changes in body composition (9,13,14). These effects of androgen deficiency may, of course, also indirectly affect skeletal homeostasis. In this regard, the observation of skeletal changes in response to androgen replacement does not have to imply direct androgen action.

Not only extraskeletal effects should be taken into account. Complex interactions of different simultaneous effects of androgen deficiency on different compartments of an immature rat skeleton should also be recognized (see also Fig. 1). Indeed, male rats almost never reach skeletal maturity in the "human sense:" their growth plate remains open during (almost) their whole life span (15). One can try to circumvent this problem by using older rats with lower longitudinal bone formation. However, the conclusion

reached by experiments in any rodent model will never be fully applicable to the human mature skeleton. Nevertheless, some remarkable similarities between the human and the rodent with respect to loss and gain of androgen action are apparent as will be indicated in this chapter.

The relative lack of Haversian remodeling of cortical bone in the rat (in comparison with human bone), should be considered in the evaluation of cortical bone (15,16).

The male orchidectomized model has many similarities with the well characterized osteoporosis model of the female ovariectomized rat (17). However, the female rat skeleton also differs from males. Indeed, female rats have less cortical bone and their growth plates close earlier (18). Moreover, females react differently to ovariectomy than males to orchidectomy: longitudinal and periosteal bone formation decrease in growing orchidectomized males (14,19,20) whereas ovariectomized female rats respond with increases of both longitudinal and periosteal bone formation (19,21). This opposite adaptation to sex hormone deficiency abolishes the sexual dimorphism of the skeleton. Therefore, a good understanding of gender differences in skeletal adaptation to sex hormone deficiency is necessary. Finally, possible differences in skeletal adaptation between different rat strains should also be considered (22).

THE MALE ORCHIDECTOMIZED RAT MODEL: A GOOD MODEL FOR OSTEOPOROSIS?

Osteoporosis may be defined as a mechanical failure of bone associated with net loss of bone mineral per unit bone volume. Neither rats nor mice, however, experience minimal trauma fractures. Therefore, the term “osteoporosis” is never fully applicable to rodents. Nevertheless, the female ovariectomized rat model is certainly a useful pre-clinical model for the early evaluation of pharmacological modalities in postmenopausal osteoporotic women (17). For instance, this model has helped us to understand how sex steroids play a role in the gain and maintenance of bone mineral, which are the cellular events associated with these gains or losses of bone, how these changes reflect in mechanical fragility and—most importantly—how can we pharmacologically interfere.

The male orchidectomized rat model, on the other hand, is a model for a very specific and uncommon form of human osteoporosis. Overt hypogonadism is not a very prevalent condition in men. Men, in contrast with women, do not experience a natural menopause. Partial testosterone deficiency is, however, found in about half of the healthy elderly men (when their bioavailable testosterone concentrations are compared with young normal testosterone ranges) (23). Therefore, the only condition in humans, fully equivalent to a sudden and complete removal of androgens (as induced by orchidectomy of rats), is surgical or chemical castration. Castration, however, is only performed in men suffering from either prostatic carcinoma or paraphilia. Therefore, the comparison of skeletal effects of androgen deficiency, observed in an orchidectomized rodent model, with the potential skeletal effects of a much more progressive and incomplete loss of testosterone observed in elderly men, is not justified (as will be demonstrated in greater detail). Only about 15% of all men with spinal osteoporosis have some degree of hypogonadism (24). The prevalence of hypogonadism in frail elderly men admitted with minimal trauma hip fractures is probably higher (25), but it remains to be clarified to what extent their sometimes very low testosterone concentrations are induced by their comorbidity. Men with idiopathic osteoporosis, on the other hand, have no associated hypogonadism by definition (26). This clinical condition is characterized by a histological picture of sluggish osteoblastic performance without significant changes in most biochemical bone turnover markers

(27,28). It is clear, therefore, that the skeletal impairment in idiopathic osteoporosis may be quite different from the skeletal changes described in orchidectomized rats.

In conclusion, the most important scientific merit of the male orchidectomized rodent as an osteoporosis model is the understanding of how androgens act to gain and maintain bone mass. Since male rats practically gain bone mass during a great part of their life span, most of the information will be relevant for our understanding of androgen action on bone gain. However, some of the information will also be relevant for the understanding of androgen effects on skeletal maintenance. Therefore, effects of androgen deficiency in fast growing younger rats and slow growing older rats will be discussed separately.

ANDROGEN DEFICIENCY IN GROWING MALE RATS

In young growing rats, bone grows by modeling. Bone modeling is the combined process of bone growth at the growth plate by endochondral bone formation, at the outer cortex by periosteal bone formation and at the inner cortex by combined endosteal bone formation and resorption (resorption being greater than formation). Simultaneously, cancellous bone grows by two forces: endochondral bone formation adds cancellous bone proximally, which is resorbed again distally. The end result is a continuous enlargement of the bone marrow cavity.

Bone modeling adapts in response to changing environmental conditions. The best characterized condition is mechanical force. Absence of mechanical strain on bone cells during growth—as occurs during immobilization—has dramatic effects: less bone will be added at periosteal, endosteal sites and at the growth plate (8,29). The bone marrow, however, does not react similarly to this offset of mechanical force: resorption goes on and the bone marrow cavity continues to enlarge. Immobilization therefore results not only in less cortical and trabecular bone, but also in thinner and shorter bones.

Skeletal features of androgen deficiency in growing male rats show some similarities with the effects of immobilization. Indeed, both periosteal and longitudinal bone formation also decrease following orchidectomy (19). Therefore, less bone is added at these sites. However, androgen deficiency (as mentioned earlier) may affect mechanical strain (and thus bone modeling) indirectly due to failure of body weight gain (9,13,19). Furthermore, not only weight gain, but also body composition may change following orchidectomy in favor of more fat and less lean body mass. Therefore, the overall decrease of bone mineral content in orchidectomy rats may be linked to a decrease of lean body mass, but this remains to be firmly established. Less muscle indeed means less strain on bone cells, which therefore will add less bone. Not only androgens, but also the growth hormone—IGF-I axis acts as a key regulator of growth. Androgens may thus affect skeletal growth indirectly via the GH–IGF-I axis. Bone modeling decreases in growth hormone-deficient male rats following orchidectomy (30). Therefore, androgens directly stimulate (at least partly) skeletal growth in male rats.

Despite similarities, there are also some differences between androgen deficiency and loss of mechanical strain. Running exercise fails to prevent or reverse either net bone loss or relative reduction of bone gain induced by orchidectomy (31). Moreover, there is now good histomorphometric evidence that, in contrast with the low periosteal bone formation and endochondral ossification, bone resorption (characterized by greater increase of resorption than formation) significantly increases at both cancellous and endosteal sites (14,20,29). This increase of bone resorption within the marrow cavity is also reflected by an increase of several biochemical markers of bone turnover (32).

The end result is a dramatic decrease of both bone mineral and size following orchidectomy (32,33). Not only bone volume and size decrease in orchidectomized rats, but also less bone mineral is added and more bone lost per volume (30). The overall decrease of bone density observed in growing orchidectomized rats is, however, only explained by an apparent decrease of cancellous bone density (reflected histomorphometrically in less and thinner trabeculae, without changes in real density of these trabeculae) (20,32,33). No real changes in cortical density (when expressed as cortical volumetric density measured by pQCT) occur in orchidectomized rats (32). Apparent density of cortical bone by DXA, on the other hand, seems lower due to decreases in cortical size (32). This lack of cortical changes probably relates to the lack of Haversian remodeling.

In conclusion, during androgen deficiency, growing male rats will add less bone. This decrease of new bone formation may be associated with overall changes in growth and body composition which are at least partly independent of the GH-IGF-I axis. However, not only will less bone be added, more bone will be resorbed as well in orchidectomized vs intact growing rats.

ANDROGEN DEFICIENCY IN AGED MALE RATS

In aged male rats, both growth rate and body weight gain are much less (15,34–36). Growth plates are still open but longitudinal growth is extremely low. Biochemical and histological bone turnover parameters show age-related decreases in these older animals. Periosteal bone formation continues, but at a much lower rate (15,34,36). Because bone marrow enlarges at a fast rate, no extra cortical bone is added (35–37). Instead, a gradual loss of cortical bone is observed. Similarly, cancellous bone volume decreases, primarily as the result of the combination of ongoing cancellous bone resorption and relatively decreased cancellous bone formation. Therefore, and despite a number of limitations, the aged male rat skeleton resembles age-related changes in elderly males (4,26). Overall, in both skeletons, the age-related loss in bone size and volume is due to a decrease of bone formation without significant increase of bone resorption.

Androgen deficiency in older rats has effects on body weight gain but body composition also changes, as evidenced by a decrease in lean body mass (38). The effects of androgen deprivation on growth plates and periosteal surfaces are considerably less than in growing rats, although a trend towards lower periosteal bone formation is evident (35,36). Androgen deficiency in older rats, however, does not induce significant changes in bone length (34,35,39,40).

The most marked skeletal response to androgen deprivation in older rats is an increase of cancellous and endosteal bone resorption resulting in cancellous bone loss (34,37,41–43). Although cancellous bone formation also increases in response to the increased cancellous bone resorption, bone formation increases less than resorption. This process is similar to the uncoupling between bone resorption and formation that occurs in bone remodeling units in early postmenopausal women (28). At the same time, more cancellous bone remodeling units are activated. As a result of this overall relatively increased endosteal and cancellous bone resorption, the marrow cavity enlarges and some thinning of the cortices occurs. Moreover, an increase of cortical porosity occurs, presumably secondary to this increase of bone turnover at the endocortical surfaces, at least in some (36,39) but not all studies (40).

Overall, net loss of cancellous bone, cortical thickness and increase of cortical porosity complete the typical skeletal picture in older androgen-deprived rats.

In men suffering from prostatic carcinoma or sexual delinquency, similar loss of cancellous bone density is observed following either chemical or surgical castration (44–

46), and this loss is associated with increased biochemical markers. Cortical changes in bone following castration are less well described in humans. Also, the histomorphometric changes following castration are unknown.

Effects of Androgen Replacement in Male Orchidectomized Rats

THE THRESHOLD ISSUE

Androgen replacement prevents all of the above described skeletal changes in both young and older orchidectomized rat models, suggesting that androgens are needed to gain and maintain cortical and cancellous bone in male rats (13,14,34,36,37,47).

Testosterone replacement has similar effects in hypogonadal men (48–50). Again, the effects of androgen replacement on cortical bone are less well evaluated in humans. According to some (51) but not all (48) studies, the effects of androgen replacement in hypogonadal men are considerably less when started after reaching maturity, suggesting that androgens are also important for building peak bone mass in men.

The role of testosterone in skeletal maintenance in hypogonadal men after growth may be of greater relevance. Indeed, 50% of healthy elderly men will experience some degree of relative hypogonadism as evidenced by a decrease of their bioavailable testosterone compared to young men (23). However, in most of these men testosterone does not fall to very low concentrations as found in men with overt hypogonadism or after castration. Therefore, the question of a threshold concentration of testosterone with respect to bone is very important. In a recent experiment, administration of a low dose of testosterone (a dose that was not able to prevent more than 50% of the loss of ventral prostate and seminal vesicles weight) prevented bone loss in orchidectomized animals (38).

These findings provide strong evidence that the threshold concentration of testosterone in aged rats is lower for bone than for ventral prostate or seminal vesicles. Interestingly, in this experiment, testosterone action on bone was strongly correlated with its effect on lean body mass (38). Not only in younger but also in older rats, a close relationship may thus exist between effects of testosterone on bone and muscle.

The combination of GH and testosterone administration in very old orchidectomized rats was not superior with respect to their effect on bone mass or density, although effects of both hormones on periosteal bone formation seemed additive (36).

ESTROGEN VS ANDROGEN RECEPTOR PATHWAY

Another issue of great pharmacological importance is whether androgens act via the estrogen receptor or androgen receptor pathway (*see* Fig. 1). Androgen action may indeed depend on direct binding on the androgen receptor (either testosterone [T] directly or dihydrotestosterone [DHT] after conversion of T via 5 α -reductase) but androgens may also act via the estrogen receptor pathway following aromatization into estrogens.

Interestingly, estrogen administration in the aged orchidectomized rat model, resulting in concentrations about twice as high as normal, prevents cancellous bone loss as the result of a dramatic reduction of bone turnover (34). Cortical changes are less pronounced in these older animals, although in younger male rats treated with estrogen, important decreases of periosteal bone turnover have been described (52). The decrease of bone turnover and preservation of cancellous bone occurs despite important side-effects such as net body weight loss, decreased body weight and earlier closure of the growth plate (34). Therefore, some of the effects of estrogen in male rats may be explained by “estrogen-induced osteosclerosis in rat” (53). Indeed, estrogen treatment decreases

resorption of calcified cartilage during vascular invasion of the growth plate, decreases at the same time resorption of cartilage and bone matrix in the primary spongiosa and further decreases bone resorption in the secondary spongiosa (54). The net result of these three processes is an increase in cancellous bone volume explaining why estrogen administration, resulting in only slightly supraphysiological estradiol concentrations, increases cancellous bone volume in orchidectomized rats above intact levels. Indeed, although growth is slow in 15 month old male Wistar rats, epiphyses are not fused (15). Therefore, although skeletal effects of estrogen are again remarkably similar to the effects of estrogen administration described in adult transsexual male to female men (low bone turnover and prevention of bone loss) (55), the mechanism of action of estrogen in an animal with nonfused epiphyses is not enterily comparable to the human situation.

In men, the estrogen pathway has received much attention because an important delay of skeletal maturation has been described in men with a mutation in the estrogen receptor- α gene (56) or in the aromatase gene (57–59). Moreover, in an aromatase-deficient man, estrogen administration was found to increase bone density at different skeletal sites, probably as a result of increased bone maturation (59).

In rats, genetic defects of either aromatase or estrogen receptor- α or - β have not been described (in contrast with mice, see further). The estrogen receptor pathway, however, was evaluated in male rats via pharmacological modulation in one of our recent experiments (*see also* Fig. 2). In this experiment, male rats were treated with a specific non-steroidal aromatase inhibitor vorozole, both at 2 mo of age (active growth) (32) and 12 mo of age (41), during a period of 15 wk. Although it is difficult to ascertain that vorozole completely blocks aromatase activity at the local tissue level in a male rat, a significant reduction of serum estradiol (measured by gas chromatography coupled with high resolution mass spectrometry) was obtained during both experiments. In the younger rats, less bone was added during administration of vorozole, both at cortical and cancellous sites (32). This decrease of bone gain was similar to the effect of castration in these animals. However, in contrast with the vorozole-treated rats, orchidectomized animals had higher bone turnover when evaluated by biochemical markers. Vorozole administration also retarded growth rates, perhaps as a result of decreased serum IGF-I concentrations. It is possible, therefore, that the observed delay of bone gain during vorozole treatment is mediated by indirect effects of the inhibitor on the GH-IGF-I axis. The higher bone turnover rates in orchidectomized animals and the trend to lower bone densities (especially when castrated rats received also vorozole) suggest additional effects of androgens via the androgen pathway. In older rats, also a decrease of cancellous bone density was observed during vorozole administration. In this model, cortical changes were not significant, although again a decrease of body weight gain and lowering of serum IGF-I was observed (41).

Moreover, a selective estrogen receptor modulator was recently shown to prevent bone loss induced both by aging and orchidectomy in adult rats (42) (*see also* Fig. 2).

In summary, pharmacological modulation of the estrogen receptor pathway has important skeletal effects in male rats. Extra estrogen clearly increases cancellous bone mineral density and decreases bone turnover, despite considerably lower overall growth rates. Moreover, extra estrogen makes a male rat more female with respect to its bones. Induction of estrogen deficiency decreases bone gain and maintenance but at the same time also interferes with the growth rate. However, in men and animals with nonfused

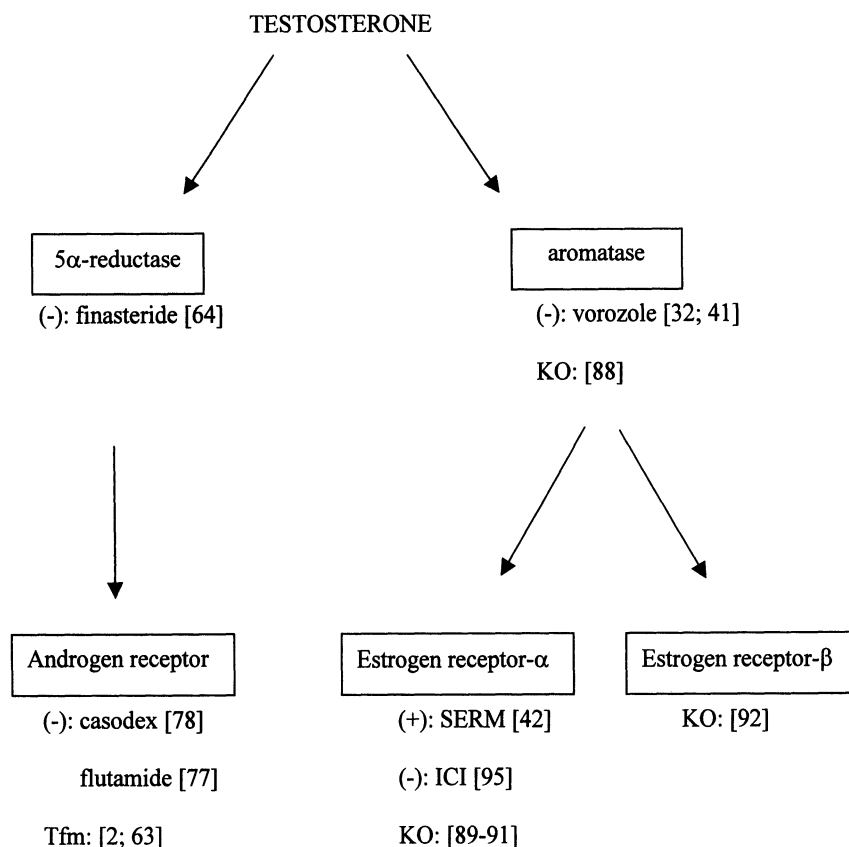


Fig. 2. Different approaches to study the androgen versus estrogen receptor pathway in rodents using (+) stimulators or (–) inhibitors of either enzymes or receptors, KO (knockout) models or natural mutants (Tfm) of receptors. References are indicated between brackets.

epiphyses, the mechanism of action of estrogen may differ from the effects of estrogen after growth. Indeed, estrogen not only closes growth plates in both rats and humans, but also increases cancellous bone volume.

The observation that both estrogen administration and deprivation act on skeletal homeostasis of male rats as well as men, at least when epiphyses are not fused, does not exclude a role of the androgen pathway. Indeed, both in younger (14,47) and older male rats (34), the nonaromatizable androgen DHT was able to prevent most of the skeletal changes induced by orchidectomy.

Another model that is useful for the study of the androgen receptor pathway is the Tfm rat (*see also Fig. 2*). The testicular feminized male rat model is the rat homologue of the human syndrome. As a result of a single transition mutation in the androgen-binding domain of the androgen receptor gene and subsequent androgen insensitivity, the affected animals develop a female phenotype (60). Case-reports of humans with a similar syndrome are inconsistent, showing either near normal or decreased bone density (1,61,62). Much of the confusion may be explained by the fact that most of these patients were orchidectomized at different ages and more or less compliant to the well-established effects of the estrogen therapy that they receive. The rat model controls for these confounders. However, it is important to realize that Tfm rats lack androgen action during

their entire life span, including in utero and neonatal androgen action. The fetal androgen action affects the GH-IGF-I axis profoundly, which may explain some of the later skeletal effects. Secondly, because of increased aromatization of their non-functional androgens, Tfm rats have significantly higher estrogen concentrations than their normal littermates. Therefore, the Tfm male rat should essentially be regarded as a male animal with loss of androgen action, lower GH-IGF-I and higher estrogen secretion.

In order to circumvent the possible confounding role of this excess estrogen action during puberty, the authors evaluated skeletal changes in both Tfm and male littermates that were orchidectomized before puberty. Both orchidectomized Tfm and orchidectomized normal male rats had significant lower cancellous bone density at maturity compared to intact Tfm and intact normal rats, respectively (2,63). This experiment suggests that, in accordance with the human data, testicular estrogen secretion (which both Tfm and normal rats lack after castration) is necessary to obtain normal cancellous bone volume. Although all the data fit with a crucial role of estrogen action for normal cancellous bone development, cortical size again shows a different response to changes in sex steroids during puberty. Cortical bone size was greatest in normal males having normal androgen action, suggesting that androgen action is required in order to obtain male bone size.

Again, androgen action may depend on indirect effect of androgens on body composition and/ or on the GH-IGF-I axis.

Pharmacological modulation of androgen action via finasteride, a drug which blocks 5 α -reductase activity, did not affect skeletal homeostasis in male rats (64) (see also Fig. 2). This parallels the lack of skeletal side-effects of finasteride in humans (65). However, finasteride is a specific type II 5 α -reductase inhibitor. Since we do not know which type of 5 α -reductase is present in bone, the lack of skeletal effects of 5 α -reductase inhibitors does not necessarily imply that 5 α -reductase activity is not involved in regulation of skeletal homeostasis.

Androgen Effects in Female Rats

Androgens seem to be important for female skeletal homeostasis as well. Indeed, hirsute women have higher BMD than nonhirsute women, even after correction for BMI which is usually higher in hirsute women (66). Moreover, androgen administration in combination with estrogen has a stimulatory effect on biochemical markers of bone turnover in postmenopausal women (67). There has been considerable interest in the potential role of the so called "weaker" androgens. Of all sex steroids, they have the highest serum concentration in humans and show the most dramatic age-related declines (68,69). However, the scientific evidence that dehydroepiandrosteronedione (DHEA) or its sulphate ester (DHEA-S) protect against bone loss in humans is weak.

Interestingly, many authors have reported similar reductions of androstenedione and DHEA following ovariectomy in female rats (70–72). Several studies in female ovariectomized rat models have confirmed that androgen action has bone-sparing effects on cancellous bone. In most of the studies, weaker androgens like DHEA or androstenedione were used (70–73) although some protective effects following administration of relatively high doses of DHT have been reported in estrogen-deficient rats (73–75).

The action of the weaker androgens depends on stimulation of the androgen receptor and not of the estrogen receptor. Indeed, concomitant administration of antiandrogens (72,73) but not of an aromatase inhibitor (71,72) or estrogen antagonist (73) blocks the bone-sparing effects of androstenedione in female ovariectomized rats. Interestingly,

cellular events following administration of weaker androgens (decrease of local osteoclast and osteoblast surfaces and bone formation rates) are similar to the effects of estrogen (70–73). However, opposite stimulatory effects of DHT on cancellous bone formation have been reported in ovariectomized rats treated with a biphosphonate (76). Therefore, at first glance, the mechanism of the weaker androgens on cancellous bone turnover (although apparently androgen receptor-related) seems similar to estrogens, whereas DHT effects appear to be different.

Androgen action on female cortical bone, on the other hand, is less consistent. Moreover, gender differences in cortical action of androgens have been reported. Indeed, DHT stimulates periosteal bone formation in growing orchidectomized rats, but decreases periosteal bone formation in ovariectomized females (47). However, this gender difference of DHT was not confirmed in estrogen treated ovariectomized rats: an increase of periosteal and longitudinal bone formation rate was reported (75). This apparent inconsistency of DHT action in different studies may be explained by either different dosing (which is difficult to evaluate since serum concentrations were not reported) or by extraskelatal effects (*see* Fig. 1). It is possible that extraskelatal DHT action on growth rate and body weight confounds some of the observed cortical effects (47,75,76).

Weaker androgens do not seem to have much effect on cortical bone since they affect neither increase of cross-sectional area, nor cortical area or longitudinal growth rate (although a modest stimulatory effect on cortical bone formation was observed in one study [70]).

Finally, one study has reported a decrease of total bone calcium in intact female rats treated with the antiandrogen flutamide (77). Results of this study are difficult to interpret because of failure to document cortical and cancellous changes in bone turnover and mass and of possible confounding effects on weight gain. Casodex, also an antiandrogen, reduced cancellous, endosteal and periosteal bone formation rates in estrogen-replete female rats, but did not induce changes in cancellous bone volume (78). Furthermore, no differences in body weights were reported (*see* also Fig. 2).

In conclusion, most studies in female ovariectomized rats have shown modest cancellous bone sparing effects of DHEA and androstenedione without significant effects on bone cortex. These effects are probably explained by a reduction of bone turnover and are related to stimulation of the androgen receptor. These effects are interesting because they already occur when concentrations of androstenedione are restored to the normal range. The relevance of DHT effects is difficult to interpret because of the high nonphysiological doses used and the possible extraskelatal side-effects.

Studies of Androgen Action in Mice

Mice are an attractive animal model for bone studies because of the possibilities of transgenic technology. However, mice also have some limitations. Gender differences in bone size seem much less pronounced in mice than rats (53). Both skeletal and extraskelatal effects of androgen deficiency are also less well established.

Both static and dynamic histomorphometric indices at cancellous sites of either the distal femur or proximal tibia clearly increase early after orchidectomy (79–82), resulting in considerable decreases of cancellous bone volume. This “loss of cancellous bone” probably occurs as a result of a relatively greater increase of bone resorption than formation although this process is probably not entirely similar to the bone remodeling

process in humans. According to some authors, castration of male and female mice results in similar cancellous events (80).

The castrated mouse model also has an important scientific merit: in mice, a clear relationship has been established between the cellular events in bone that lead to increase of both bone resorption and formation on the one hand and the cellular changes and their regulation in bone marrow cells on the other hand (80,82–84). Indeed, the critical role of the bone marrow stromal cell as a key regulator of bone turnover was demonstrated in mice. Interestingly, the cellular changes in bone and bone marrow that occur postovariectomy or -orchidectomy are completely similar. Both procedures lead to an upregulation of CFU-GM (dependent on increased secretion of M-CSF by bone marrow stromal cells) and increased differentiation of these cells into osteoclasts. *In vitro*, both androgens and estrogens prevent these increases of cytokines that mediate the upregulated osteoclastogenesis and subsequent bone resorption.

The crucial role of these bone marrow stromal cells, which are osteoblast precursor cells, in the regulation of osteoclastogenesis was demonstrated in SAMP6 mice (81). These SAMP6 mice are a model of accelerated senescence and osteopenia. They show an age-related decrease in osteoblast progenitors which in turn leads to defective bone formation associated with reduced bone mineral density. Orchidectomized SAMP6 mice will develop neither increase of cancellous bone turnover nor extra cancellous bone loss (82). This defect in osteoclastogenesis is secondary to impaired osteoblast formation and can easily be restored by addition of osteoblastic cells from normal mice to marrow cultures of SAMP6 mice.

Therefore, the removal of sex steroids by castration in mice (in both male and female) primarily leads to bone marrow changes. These changes, mediated by cells of the osteoblast lineage, are characterized by an increase of osteoblast precursors which in turn indirectly stimulate the now well documented osteoclastogenesis. Increased osteoclast activity and decreased osteoclast apoptosis complete the picture (85).

The SAMP6 mice model may also be of relevance for humans. Indeed, SAMP6 mice show striking similarities with human senile osteoporosis. Men lose bone primarily as a result of defective bone formation (4). Therefore, the acute and complete androgen withdrawal induced by orchidectomy has clearly different cellular effects in both bone and marrow. In concordance with these findings, men suffering from idiopathic osteoporosis seem to have low bone turnover (27) in contrast with hypogonadal osteoporotic men who tend to have higher cancellous bone turnover (86).

Transgenic technology has created the opportunity to better study the relevance of the estrogen receptor pathway for male skeletal homeostasis (Fig. 2). However, reports are still limited and unfortunately mostly published in abstract form.

The androgen receptor pathway seems involved in early development of sexual dimorphism of the mouse skeleton (87). Adult aromatase-deficient mice (ArKO), however, have an osteoporotic phenotype with low bone turnover rates (88). This finding may also be related to overall changes in growth (although body weight changes have not been reported), because male ArKO mice also have decreased femur length. In accordance with ArKO mice, male estrogen receptor- α knockout mice (α ERKO) also have smaller and shorter bones compared to wild type littermates (89–91), which may be explained by their lower serum IGF-I levels (90). Cancellous bone mass, however, is increased in male α ERKO mice and responds to androgen deprivation and replacement (91). Also, femurs of male α ERKO mice appear to be shorter. In contrast, there are no differences between male estrogen receptor- β knockout mice (β ERKO) and age-matched wild types (92).

In summary, the role of the estrogen vs androgen receptor pathway in male skeletal homeostasis remains also unclear in mice (79). In this regard, it should be noted that skeletal changes induced by orchidectomy in male mice can be prevented not only by dihydrotestosterone (93) but also by estrogen and raloxifene, a selective estrogen receptor modulator (94).

CONCLUSION

Despite a number of limitations, animal studies have increased our general understanding of the putative skeletal role of sex steroids. Animal data indicate that androgen action is involved in gain and maintenance of the male skeleton. It is difficult, however, to establish the extent to which this androgen action requires direct stimulation of bone cells. In fact, androgen action on cortical bone seems closely linked to lean body mass (probably independent of the GH-IGF-I axis effects on bone or lean body mass).

Contrary to the growth promoting effects of androgens, estrogen is a growth limiting hormone in both sexes (at least in higher concentrations). Estrogens, however, result in extra (cancellous) bone mineral in both sexes. Removal of sex steroids induces similar cellular events in cancellous bone and marrow in both sexes. Moreover, molecular regulation of these cellular events seems similar in both sexes. Cancellous and cortical bone respond to androgen and estrogen receptor stimulation in both sexes. The relative role of both receptor pathways, however, remains to be clarified.

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19

Gonadal Steroids and the Skeleton in Men

Clinical Aspects

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INTRODUCTION

Osteoporosis has long been recognized as a major cause of morbidity and mortality in the elderly, especially in women. Because of its prevalence and clinical importance, most of the research, therapeutic, and educational efforts have focused on postmenopausal osteoporosis. In 1940, Fuller Albright proposed that estrogen deficiency was the causative factor in postmenopausal osteoporosis, the first suggestion that gonadal steroids play an essential role in bone metabolism (1). Albright and colleagues then demonstrated that estrogen administration reverses the negative calcium balance associated with estrogen deficiency and that both estrogens and androgens induce positive calcium balance in estrogen deficient osteoporotic women and eugonadal osteoporotic men (2). Based on these early observations and accumulating experimental data, estrogen replacement soon became a powerful therapeutic agent in the treatment of postmenopausal osteoporosis.

The clinical importance of osteoporosis in men is now receiving more attention from clinicians and researchers. This new awareness likely corresponds to the extension of average life span in both sexes and changing demographics in the developed world (3). Based on the World Health Organization femoral bone mineral density (BMD) definitions of osteoporosis and osteopenia, and a male reference base, a recent study found that 3–6% (1–2 million) of non-Hispanic white American men 50 yr of age or older have osteoporosis and 28–47% (8–13 million) have osteopenia (4). If the same analysis is

performed using a female reference base, 1–4% (280,000–1 million) of men would be classified as osteoporotic and 15–33% (4–9 million) classified as osteopenic (4). Thus, while the prevalence of osteoporosis and osteopenia in older men is less than in women, it remains a common condition that can lead to significant clinical consequences including hip fracture—30% of which occur in men (5).

Gonadal steroids play a key role in bone metabolism throughout life in men. The sharp rise in serum testosterone (and estradiol) levels that occurs at puberty is associated with a dramatic increase in bone mineral content and density. Conversely, aging in men is associated with both a decline in adrenal and testicular androgens and a progressive decrease in both trabecular and cortical BMD. Furthermore, hypogonadism is a well-recognized and treatable risk factor for osteoporosis and fracture in men. Within the past decade, intensive in vitro and animal investigation has begun to unravel the molecular and cellular mechanisms that underlie these clinical observations. Many of these basic studies have focused on the interactions of androgens and estrogens with their respective receptors on osteoblasts and bone marrow stromal cells and the subsequent regulation of bone resorbing cytokines and other local factors. An in-depth discussion of these issues is provided in previous chapters and provides a framework for understanding the clinical effects of gonadal steroids on the skeleton in men.

In this chapter, we will review the effects of gonadal steroids on bone metabolism in men as well as the epidemiological data concerning the relationship between circulating gonadal steroid levels and BMD. We will then review the skeletal consequences of various disorders of gonadal steroid production, metabolism, and action in men, as well as the therapeutic effects of gonadal steroid administration. Finally, we will give special attention to an emerging fundamental question in male bone biology—the differential roles of androgens and estrogens in skeletal development and homeostasis.

EFFECTS OF GONADAL STEROIDS ON BONE METABOLISM IN NORMAL MEN

Adult BMD is determined by two factors: (1) the peak bone mass achieved during development and (2) the amount of bone lost after accrual of peak bone mass. Because gonadal steroids affect both of these processes, they modulate bone metabolism throughout life in men.

Skeletal Changes at Puberty

Puberty is associated with dramatic increases in cortical and trabecular bone in both boys and girls (6). In the axial skeleton, peak bone mass is reached by approximately age 20 in males (7–9), whereas peak bone mass in the appendicular skeleton is reached somewhat later (10). The extent to which differences in the adult male and female skeletal development are due to differences in pubertal gonadal steroid secretion or other genetic or environmental factors is unknown. It is clear, however, that the sharp rise in serum testosterone (and estradiol) levels that occurs at puberty in males precedes the increase in biochemical markers of bone turnover (11,12), and the marked pubertal increase in cortical and trabecular BMD (7–9,12). Bonjour et al. (Fig. 1) measured BMD by dual X-ray absorptiometry (DXA) and found that BMD correlates with pubertal stage in trabecular (lumbar spine), mixed (femoral neck), and cortical sites (femoral shaft) in boys and girls (8). Furthermore, exogenous testosterone enanthate administration (a

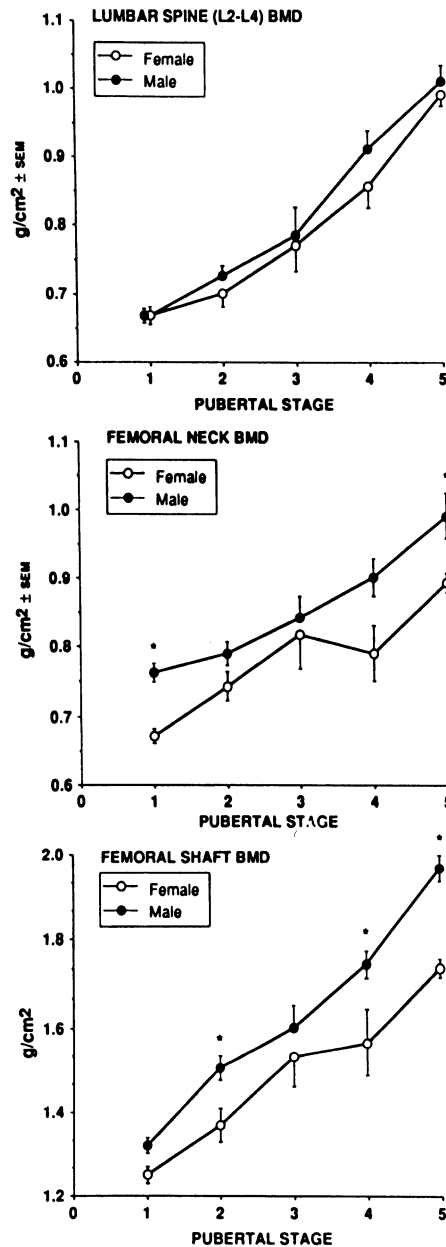


Fig. 1. Relation between BMD of the lumbar spine, femoral neck, and femoral shaft and pubertal stages in female and male subjects. * $P < 0.05$ for comparison between male and female. Reproduced with permission from ref. 8.

testosterone ester that can be aromatized to estrogens) promotes calcium retention in prepubertal boys (13). The specific effects of nonaromatizable androgens on bone mass and calcium balance during this critical period of skeletal development, however, are not well defined.

The most striking difference observed in the skeletons of men and women is in bone size and total bone mineral content (BMC) (14). Vertebral body size is larger in males than in females beginning at an early age (15). Cancellous bone density, however, is not

higher in males compared with females, especially after corrections are made for height, weight and stage of puberty (15,16). In fact, a recent cross-sectional study of young African-Caribbean and Caucasian adults reported that lumbar spine BMD was similar in men and women (17). In the same study, bone mineral apparent density (BMAD), an estimated volumetric bone density that corrects BMD measurements for bone size (necessary because of projection errors introduced by DXA measurements), was actually greater in women (17).

Bone size and BMC in the appendicular skeleton (predominantly cortical bone) is also greater in males than in females and males appear to have greater cortical BMD when measured by standard absorptiometric techniques as well (8). It is unclear, however, whether these gender differences persist, or are reduced, when cortical BMD measurements are corrected for bone size (17–19). The greater cortical bone mineral content of males has long been hypothesized to be due to specific effects of androgens. The extent to which the higher cortical BMC in men is related to differences in gonadal steroid secretion also remains unclear. In fact, a recent study examined this question in a cross-sectional study of 375 healthy young adults and found that the increase in midfemoral bone mass occurring during adolescence is primarily related to increases in mechanical loading (as reflected by body mass) as opposed to gender (20). Thus, if androgens play a specific role in increasing cortical bone mass in men, they may be acting indirectly by increasing body mass via their anabolic effects on other tissues. Taken together, these observations, as well as the emerging understanding of the importance of estrogens in skeletal development in men (discussed below), suggest that androgens may be only one of several important modulators of the accrual of peak bone mass in men.

Gonadal Steroids and Skeletal Changes in Aging

Aging in men is associated with a decline in both adrenal and testicular androgens (21–24), as well as a decline in serum estradiol levels (24–27). Aging in men is also associated with a decline in both trabecular and cortical BMD (4,28–32), and a decrease in the rate of bone formation (29,33). Thus, some investigators have hypothesized that the decline in androgens in men contributes to the age-related reduction in bone formation and bone loss. Importantly, however, aging in men is associated not only with a decline in gonadal steroids, but with a variety of alterations in the action or metabolism of many hormones affecting calcium metabolism including parathyroid hormone, 1,25-dihydroxyvitamin D, growth hormone, and IGF-1 (34). Moreover, there are complex interactions between gonadal steroids, calcium regulatory hormones and the GH/IGF-1 axis. For example, testosterone administration increases serum 1,25-dihydroxyvitamin D levels in hypogonadal, osteoporotic men (35), and gonadal steroid deprivation in elderly men increases the skeletal sensitivity to exogenous parathyroid hormone infusion as measured by biochemical markers of bone resorption (Fig. 2) (36). Thus, if the declining gonadal steroid levels are contributing to bone loss in elderly men, they may be doing so by either directly affecting local modulators of bone turnover, altering sensitivity to or production of other calcium regulatory hormones, or both.

Several cross-sectional studies have examined the relationship between gonadal steroid levels and BMD in adult and elderly men. The results of these studies have been inconsistent. While some studies have reported a significant positive association between serum androgen levels (either total, bioavailable, or free) and BMD (37–40), others have found no such relationship (41–44). In fact, several studies have instead found that serum

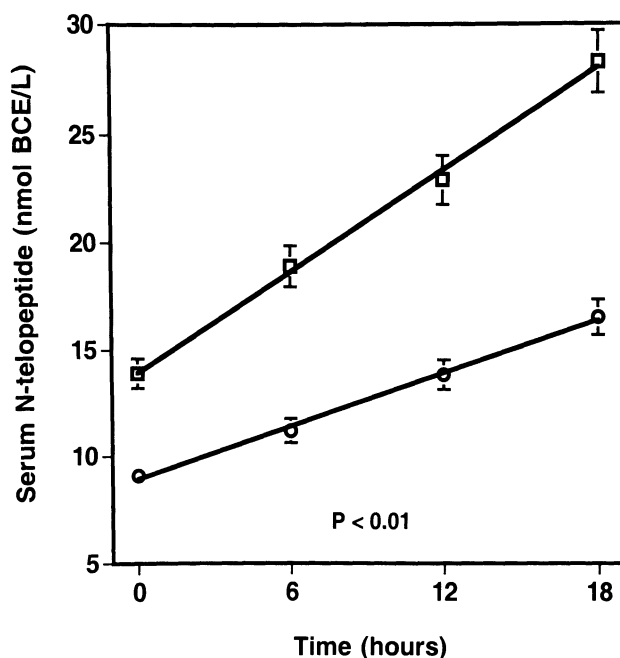


Fig. 2. Mean \pm SE serum N-telopeptide levels during h-PTH-(1-34) infusion before (circles) and 6 mo after (squares) GnRH agonist-induced gonadal steroid suppression in elderly men. The P value refers to the difference in rates of change before and after GnRH agonist administration. Reproduced with permission from ref. 36.

estrogen levels are associated with BMD at a variety of measured sites (27,43,44). Moreover, lower estradiol levels, but not lower androgen levels, are associated with vertebral fractures in men (45). These epidemiological data are difficult to interpret, however, both because of the discordant results and because of the questionable physiological relevance of associations between single hormone levels and BMD (a variable that represents the cumulative effects of a lifetime of bone accrual and resorption).

GONADAL STEROIDS AND BONE TURNOVER

It is well accepted that hypogonadism is associated with osteopenia (46), but the pattern of hypogonadal bone loss is less clear. Studies assessing the rates of bone turnover in men with hypogonadism have yielded inconsistent results. These inconsistencies may be due to the heterogeneity of male hypogonadism (both in terms of etiology and duration) and the prevalence of concomitant conditions (such as hypovitaminosis D) in hypogonadal patients. In relatively pure models of acute gonadal steroid deprivation, such as in GnRH analog-induced hypogonadism, bone turnover is increased in a manner resembling the pattern observed in early postmenopausal women (Fig. 3) (36,47–49). Similar results have been reported in men who have undergone castration (50). This increase in bone turnover is consistent with data from animal models of hypogonadal bone loss. In mice, for example, orchidectomy increases osteoclastogenesis and bone turnover (51). Moreover, this increase can be prevented (in an IL-6 dependent fashion) by administration of either aromatizable or non-aromatizable androgens (51).

Several studies have examined the effects of androgen administration on bone turnover in both normal and hypogonadal men. In normal young men, pharmacological

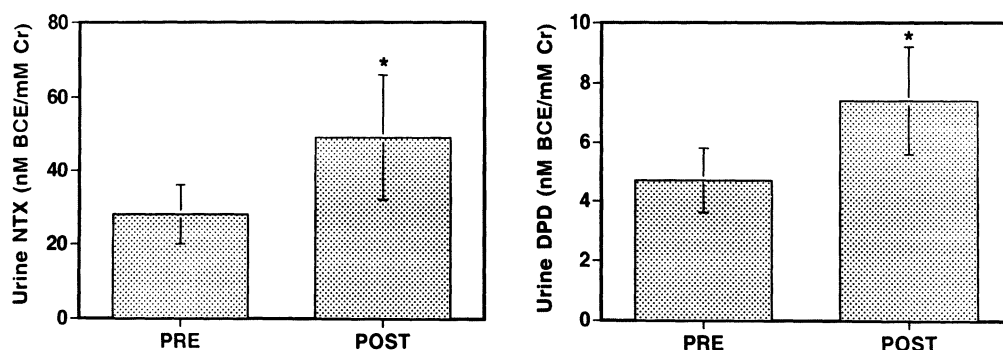


Fig. 3. Urinary excretion of N-telopeptide (NTX) and deoxypyridinoline (DPD) before (PRE) and after (POST) 6 mo of GnRH analog induced hypogonadism in 11 men ages 50–82 with locally advanced prostate cancer. * $P < 0.001$. Reproduced with permission from ref. 36.

doses of testosterone (testosterone enanthate 200 mg/wk IM for 6 mo), increase serum osteocalcin levels with no effect on urinary hydroxyproline excretion (52). In contrast, a modest dose of testosterone reduces bone resorption and formation markers in middle aged osteoporotic men (53). In a 3-yr study of elderly men with mildly low or low-normal serum testosterone levels, transdermal testosterone administration did not alter markers of bone turnover (54), although intramuscular testosterone reduced bone resorption significantly in a short-term study with a similar population (55).

In young men with primary or secondary hypogonadism, physiological testosterone replacement decreases markers of bone formation and resorption significantly (56). Conversely, in a similar group of patients, supraphysiological doses of testosterone increase bone formation markers (57). Taken together, these findings suggest that physiological doses of testosterone inhibit bone resorption while pharmacological doses, or doses that cause transiently elevated levels of testosterone (such as in IM preparations), may also stimulate formation. Additionally, because the testosterone preparations administered in these studies are also converted to estrogens, it is impossible to determine whether observed effects on bone turnover are related to androgen action, estrogen action, or a combination of the two.

BONE DENSITY IN MEN WITH DISORDERS OF ANDROGEN METABOLISM OR ACTION

Hypogonadism is present in 15–36% of men with documented osteoporosis (58,59) and elderly men with hypogonadism are 6.5 times more likely to have a minimal trauma hip fracture than are eugonadal elderly men (60). Thus, conditions of gonadal steroid deficiency, though diverse, underscore the integral importance of normal gonadal function in male bone integrity. Furthermore, the study of these conditions provides a fertile area to increase our understanding of the effects of gonadal steroids on the skeleton.

Bone Density in Men with Primary Hypogonadism and Klinefelter's Syndrome

Men who have undergone castration frequently develop osteoporosis and fracture (50,61). In a retrospective study of 235 men with prostate cancer, BMD of the femoral

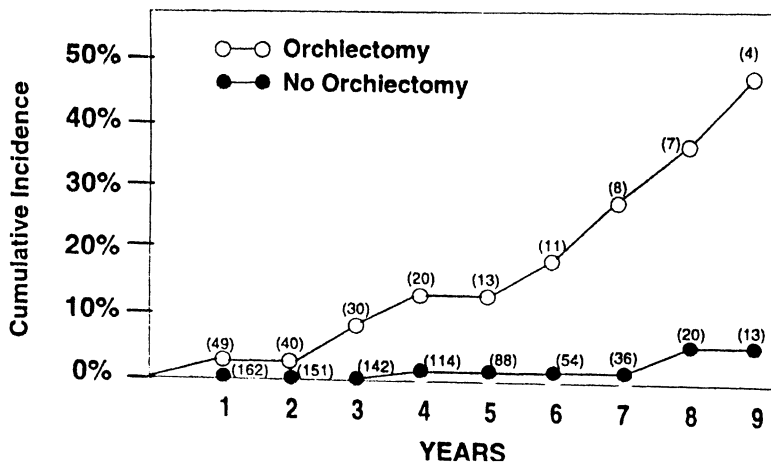


Fig. 4. Cumulative incidence of first osteoporotic fracture in men with prostate cancer with and without orchiectomy. Intervals are times from castration to last follow-up or to first osteoporotic fracture and from diagnosis, respectively. Numbers in parentheses indicate men evaluated at each interval. Reproduced with permission from ref. 61.

neck was 13% lower in men who underwent castration versus those who did not (61). Additionally, the cumulative incidence of a first osteoporotic fracture was increased more than five fold in patients who underwent castration (Fig. 4) (61). Osteopenia is also observed in hypogonadal patients with hemochromatosis, though these patients may have either primary or secondary hypogonadism (62).

Studies investigating skeletal abnormalities in men with Klinefelter's syndrome (KS) have produced mixed results. Several reports have concluded that patients with KS have reduced BMD (63–65), although a more recent study found that they have normal bone mass (66). Reports of BMD in these men may be confounded by the heterogeneity in the degree of hypogonadism, the relatively higher estrogen/androgen ratio in patients with KS, and the possible effects of the underlying genetic abnormality.

Bone Density in Men Taking GnRH Analogs

Men taking GnRH analogs for the treatment of prostate cancer are a valuable model to explore the effect of severe secondary hypogonadism on bone mass. GnRH analogs are potent inhibitors of the hypothalamic-pituitary-gonadal axis and men with prostate cancer are often treated with these drugs for many years. Several studies have reported that GnRH analogs alone or GnRH analogs combined with anti-androgens reduce BMD in men (47–49). For example, in a small prospective study, 10/17 men taking the GnRH analog decapeptyl for benign prostatic hyperplasia and urinary obstruction lost significant bone mass within 1 yr (Fig. 5) (49). In another study, the co-administration of a GnRH analog with an anti-androgen for 6 mo decreased lumbar spine and femoral neck BMD decreased by more than 6% (47). Finally, in a retrospective study of men with prostate cancer, the use of GnRH analogs was associated with a higher risk of fracture (67).

Bone Density in Men with Hyperprolactinemic Hypogonadism

Men with hyperprolactinemia often display varying degrees of gonadal steroid deficiency and thus provide a model of potentially reversible adult onset hypogonadism.

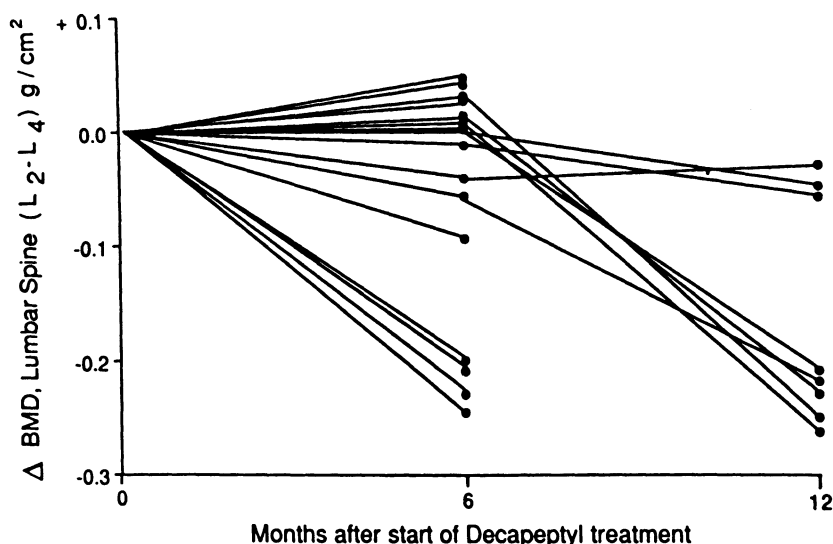


Fig. 5. Individual changes in lumbar spine BMD during 6–12 mo of decapeptyl treatment. Reproduced with permission from ref. 49.

Greenspan et al. reported that both cortical and trabecular bone mineral density are reduced in men with hyperprolactinemic hypogonadism compared to age-matched controls (68). Furthermore, cortical bone mineral density was negatively associated with the duration of hyperprolactinemia (68). In a separate study, the same group of investigators monitored bone density for 6–48 mo in these men while they were treated medically, surgically, or with radiation therapy. In men whose testosterone levels normalized, cortical bone density, but not trabecular bone density, increased significantly (69). While these findings suggest that hyperprolactinemic hypogonadism leads to loss of bone in men, it is also possible that other factors (such as growth hormone deficiency or glucocorticoid therapy) contribute to the bone loss in these patients. Furthermore, it has been reported that parathyroid hormone-related peptide (PTHrP) levels are elevated in patients with hyperprolactinemia and are inversely associated with lumbar spine BMD, suggesting that PTHrP may also play a role in the osteopenia associated with prolactin secreting adenomas (70).

Bone Density in Men with Idiopathic Hypogonadotropic Hypogonadism

Idiopathic hypogonadotropic hypogonadism (IHH) is a congenital abnormality characterized by the inability to secrete gonadotropin-releasing hormone (GnRH). Men with this disorder are hypogonadal from birth but do not have other hormonal abnormalities. As such, these patients provide a useful model to explore the role that gonadal steroids play in skeletal development during childhood and puberty as well as in skeletal homeostasis later in adulthood.

Finkelstein et al. (71), assessed cortical and trabecular BMD in adult GnRH deficient men. Men whose epiphyses were still open were compared with adolescent controls whereas men with fused epiphyses were compared with age-matched adults. Both cortical and trabecular bone density were markedly reduced in these patients and the osteopenia was similar in the men with fused or open epiphyses (Fig. 6) (71). When these men were rendered eugonadal for 2 yr (using pulsatile GnRH, hCG, or testosterone) cortical BMD increased only slightly in those with closed epiphyses (72). In contrast,

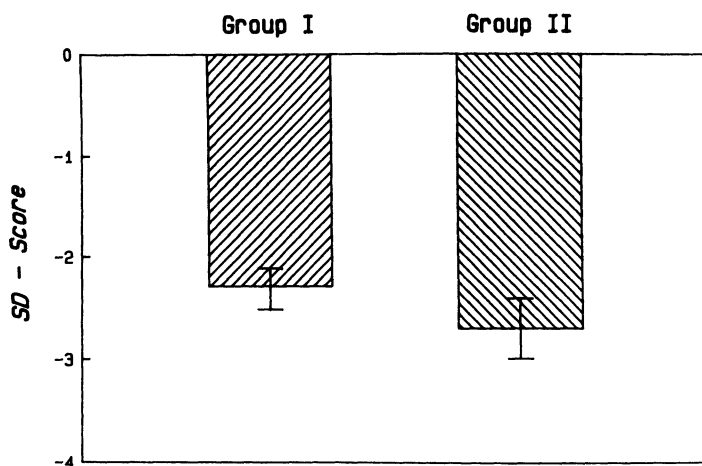


Fig. 6. Deviations of radial bone density from normal (SD-scores) in men with IHH with fused epiphyses (group I) and with open epiphyses (group II). The cortical osteopenia in these two groups was equally severe ($p = 0.45$). Reproduced with permission from ref. 71.

both trabecular and cortical BMD increased in men with open epiphyses and the increase in cortical BMD was greater in the subjects with open epiphyses than in those with closed epiphyses (though BMD still remained well below normal) (72). Furthermore, another study of BMD in treated IHH patients recently reported that the severity of the osteopenia is negatively associated with age of initial therapy (73). Together, these data suggest the primary skeletal defect in patients with IHH is related to the accrual of peak bone mass rather than to post pubertal bone loss and that there is a critical period in development when gonadal steroid secretion must occur to achieve normal peak bone mass.

Bone Density in Genetic Males with Androgen Insensitivity

Patients with complete androgen insensitivity syndrome (cAIS) are genetic males who lack androgen receptor activity. These patients are phenotypically female but have male internal reproductive organs and their serum testosterone and estradiol levels are elevated compared with normal males. Because they are completely unresponsive to androgens, these patients provide a valuable model to assess the specific effects of lifelong androgen deficiency on the accrual of peak bone mass. The model is far from perfect, however, as elevated estradiol levels prior to gonadectomy, differences in the timing of gonadectomy and variations in treatment confound published observations.

Marcus et al. (74) measured BMD in 28 women with AIS (22 complete and 6 high-grade partial), aged 11–65. In subjects with complete AIS, the mean lumbar BMD was reduced when compared with age-matched normal women though hip BMD was similar. BMD was normal in subjects with partial AIS. Women who reported good compliance with estrogen therapy had higher BMD than those who had been less compliant. When “bone mineral apparent density” (a calculated measure that adjusts for differences in bone size) was used as the primary variable, however, even estrogen compliant women had reduced spine and hip BMD compared to age-matched women. Furthermore, when BMD in these patients was compared to male standards, the reductions were even more profound. Together, these findings suggest that the osteopenia in women with cAIS

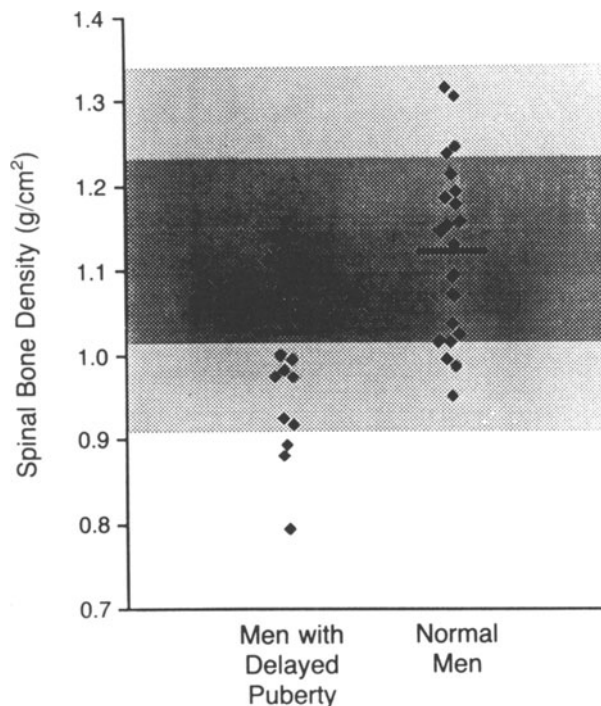


Fig. 7. Spinal BMD in 23 men with a history of CDP and 21 normal men. The horizontal lines indicate group means, and the shaded areas the mean + 1 SD and + 2 SD for the normal men. Difference between groups, $P < 0.003$. Reproduced with permission from ref. 75.

may be due not only to incomplete estrogen therapy after gonadectomy, but also to a specific role of androgen action in skeletal accrual.

Bone Density in Men with Histories of Delayed Puberty

As noted above, the observation that BMD did not normalize after gonadal steroid replacement in men with IHH suggested that there may be a critical time in development in which gonadal steroid secretion is necessary to achieve normal peak bone mass. Men with histories of constitutionally-delayed puberty (CDP) provide a useful model to test this hypothesis. In an initial study, radial BMD (assessed by SPA), spinal BMD (assessed by DXA), and femoral BMD (assessed by DXA) were reduced in men with histories of CDP who eventually underwent pubertal development without having received hormone therapy (Fig. 7) (75). However, when spinal BMD values are adjusted for the smaller size of the bones in men with histories of CDP, one group reported that BMD is similar in men with histories of CDP and controls (76), while another group reported that the reductions in BMD persist (77). Thus, it appears that the normal timing of gonadal steroid secretion at puberty may be necessary to achieve peak bone, though part of the observed BMD differences could be related to bone size.

Bone Density and the Role of Dihydrotestosterone

In many target tissues, such as skin and prostate, the actions of testosterone are substantially mediated by its conversion to dihydrotestosterone (DHT) via the enzymes 5α -reductase types I and II. A potential role for DHT in bone metabolism has been

suggested by several observations. Testosterone can be converted to DHT within human bone (78), and DHT stimulates osteoblast proliferation in several in vitro models (79). Whether there is an abnormal skeletal phenotype in men with 5 α -reductase deficiency, however, is unknown. Over the past several years, the use of the finasteride, a specific 5 α -reductase type II inhibitor, to treat benign prostatic hyperplasia (BPH) has become commonplace. In a 12-mo randomized double-blinded controlled trial of men with BPH who received either 1 mg of finasteride, 5 mg of finasteride, or placebo daily, bone mineral density did not change (80). Similarly, a separate study reported that both BMD and bone turnover markers were similar in finasteride-treated patients and controls despite mean DHT levels that were reduced by 81% in the treated patients (81). Thus, while the available data suggest that DHT may not play a crucial role in male bone homeostasis, it remains possible that more complete suppression of DHT, or inhibition of 5 α -reductase type I, would uncover skeletal abnormalities that have thus far not been identified.

THE EFFECTS OF ANDROGEN THERAPY ON BONE DENSITY IN MEN

Pharmacological doses of androgens can have profound effects on various aspects of body composition, including bone mineral density (52,82). The effects of physiological gonadal steroid replacement on BMD, however, is often more subtle. In fact, studies that have assessed the effects of gonadal steroids on BMD (as well as on bone turnover markers—as discussed above) have reported somewhat differing results. The reasons for these differences are not always obvious but may include:

- Differences in the degree of hypogonadism in the study populations.
- Differences in the timing of the onset of the hypogonadism in the study populations.
- Differences in the previous treatment status of the subjects.
- Differences in the prescribed androgen regimen from study to study.

Effects of Androgen Replacement on Bone Mineral Density in Hypogonadal Men

Most studies of androgen administration in hypogonadal men have reported beneficial effects on BMD (56,72,83,84). Androgen replacement increases BMD in men with IHH, (particularly in those with open epiphyses) (72) and in hypogonadal men with hemochromatosis (84). In men with acquired primary or secondary hypogonadism, 100 mg of testosterone enanthate administered weekly for 18 mo increased lumbar spine, but not radial, BMD (Fig. 8) (56). In a larger group of men with either congenital or acquired hypogonadism, testosterone replacement (given in a variety of preparations) also increased lumbar spine BMD (83). Furthermore, the increases in BMD were greater (39 vs 15% as measured by QCT) in men who had not previously received androgen therapy and most of the BMD increases occurred in the first year of therapy (83). Thus, testosterone replacement appears to increase BMD in frankly hypogonadal men.

Effects of Androgens on Bone Density in Elderly Men with Relative Hypogonadism

The effect androgen replacement on BMD in elderly men with borderline or modest hypogonadism (i.e., older men with testosterone levels in the low normal or mildly low range) is currently an area of great interest. Preliminary studies suggested that testosterone supplementation in this population may increase bone formation and reduce bone resorption (55,85). More recently, Snyder et al. studied the skeletal effects of transdermal testosterone in a 3-yr study of 108 men elderly men (54). Men were eligible for enroll-

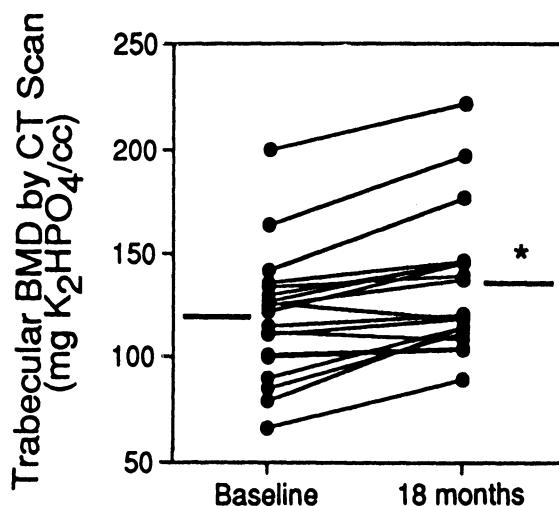


Fig. 8. Trabecular BMD determined by QCT at baseline and after 18 months of testosterone replacement therapy in hypogonadal men. The horizontal lines refer to mean values. * $P < 0.001$ compared to the baseline. Reproduced with permission from ref. 56.

ment if their serum testosterone level was less than 475 ng/dL and their lumbar spine BMD was below the mean for healthy controls. Thus, many enrolled subjects were not frankly hypogonadal and the mean pretreatment serum testosterone concentration was within the normal range (367 ng/dL). Despite a near doubling of mean total and free testosterone levels in treated subjects, there was no difference in the amount of change in lumbar spine or proximal femur BMD in testosterone-treated men vs untreated controls. A secondary linear regression analysis, however, did suggest that spine BMD improved more in treated subjects with the lowest baseline testosterone levels. Thus, the role of testosterone replacement in elderly men with mild hypogonadism remains unclear. Finally, a recent study demonstrated that alendronate therapy increases BMD in men with idiopathic osteoporosis, even in the presence of low testosterone levels (59). This finding suggests that bisphosphonate therapy may increase BMD in elderly men with modest hypogonadism. Coupled with evidence demonstrating that pamidronate prevents GnRH analog-induced bone loss (47), it appears that bisphosphonate administration may emerge as a viable, nonhormonal, therapy in the treatment and prevention of hypogonadal bone loss in men.

ESTROGEN AND BONE METABOLISM IN MEN

For many years it was assumed that androgens were the primary regulators of bone metabolism in men. Recently, however, the role of estrogens in male bone metabolism has become a fundamental question in bone biology. Not only have several epidemiological studies found that estrogen levels are associated with BMD in men (discussed above), but in vitro and animal data are also beginning to provide increasing evidence supporting a crucial role for estrogens in male skeletal development and maintenance. A large portion of what is known about this issue, however, comes neither from large cross-sectional studies nor the laboratory, but rather from the detailed observations made in a small number of men with specific genetic mutations. These patients, whose phenotypes are characterized by either an inability to synthesize estrogens or an inability to

Table 1
Clinical Characteristics in Men with Aromatase Deficiency

Report	Age	Height	Estradiol	Testosterone	LH	Osteocalcin	Lumbar spine BMD
Morishima et al. (87)	24	204 cm	<7 pg/mL	2015 ng/dL	26.1 IU/L	19.8 ng/mL (normal 3–13)	0.93 g/cm ² (T = –1.7)
Carani et al. (88)	31	187 cm	<10 pg/mL	390 ng/dL	8.9 IU/L	9.7 ng/mL (normal 5–18)	0.93 g/cm ² (T = –1.7)

respond to estrogens, have been instrumental in stimulating a reappraisal of the role of estrogens not just in bone metabolism, but in all male physiology.

Smith et al. described a 28-yr old man with a mutation in the estrogen receptor- α (ER- α) gene resulting in estrogen resistance (86). The patient had markedly elevated serum estrone and estradiol levels (145 and 119 pg/mL, respectively), normal serum concentrations of total and free testosterone, tall stature (204 cm), and continued linear growth. His skeletal phenotype was further marked by incomplete closure of the epiphyses, severe osteopenia (LS spine T score of –3.1 despite increased vertebral size), and elevations in biochemical markers of both bone formation and resorption. Additionally, pharmacological estrogen administration had no measurable effect on any of these parameters.

Two men with mutations in the gene encoding the aromatase enzyme have been described (87,88). These patients also exhibited tall stature, incomplete closure of the epiphyses, and osteopenia. Further characteristics of these patients are described in Table 1.

The reason for the differing testosterone concentrations in these two patients is unclear, but may relate to additional pituitary abnormalities in the patient described by Carani et al. In both of these patients, estrogen replacement (either transdermal estradiol or oral conjugated estrogens) induced skeletal maturation with termination of growth, as well as dramatic increases in BMD (88–90). For example, in the patient originally described by Morishima et al, the administration of 0.3 mg/d of Premarin for 12 mo followed by 0.75 mg/d for an additional 24 mo increased lumbar spine BMD by more than 20% (Fig. 9) (89). The phenotypes of these men demonstrate that estrogens are necessary for normal epiphyseal closure and strongly suggests that estrogens are essential in attaining normal peak bone mass in men.

Several experimental models have been used to address the issue of the role of estrogen in male skeletal maintenance. When anastrozole, a potent aromatase inhibitor, was administered to eight young men for 10 wk, no changes in bone turnover were observed even though estradiol levels decreased by nearly 50% (91). This model is confounded, however, by concomitant increases in serum testosterone levels in these patients. These increases are likely to be secondary to anastrozole-induced impaired estrogenic feedback at the pituitary and hypothalamus. To circumvent this problem, Falahati-Nini et al. (92) administered a GnRH analog, an aromatase inhibitor, transdermal testosterone and transdermal estradiol to 59 elderly men for three weeks and then selectively removed various agents to induce testosterone deficiency, estradiol deficiency, or combined testosterone and estrogen deficiency for an additional 3 wk. Urinary N-telopeptide increased in the men who were deficient in testosterone alone, estradiol alone, or both testosterone and estradiol. Deoxypyridinoline increased only in the absence of both hormones or in

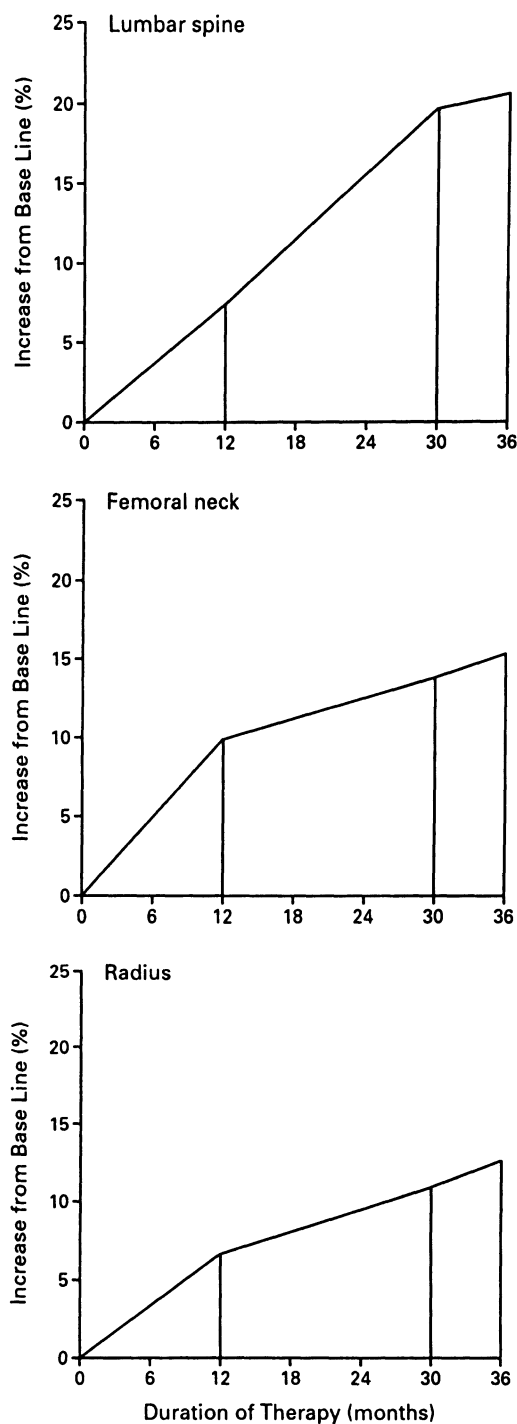


Fig. 9. Changes in BMD during estrogen therapy. The percentage increase from baseline values is shown for each site. Reproduced with permission from ref. 89.

the absence of estradiol alone. When the data were analyzed by two-factor ANOVA, estradiol appeared to play a major role in preventing the increase in bone resorption markers while testosterone did not. Nonetheless, the fact that urinary N-telopeptide

increased in subjects who were selectively testosterone deficient suggests that androgens may also be involved in maintaining normal bone turnover in elderly men. Finally, estradiol has been administered to men undergoing male-to-female gender reassignment. Although such studies are confounded by multiple variables, including the pharmacological nature of the estrogen administration, increases in spinal bone density and suppression of bone turnover, even in the face of markedly low testosterone levels, have been consistently reported (93,94). Thus, when taken together, the data appear to suggest an integral role for estradiol in both skeletal accrual and skeletal maintenance in men, though a significant and important role for testosterone in both of these processes cannot be excluded.

SUMMARY AND FUTURE DIRECTIONS

Though some of the data presented above are conflicting in specific areas, several conclusions are clearly supported. Gonadal steroids play an integral role in male skeletal development and maintenance. Boys who lack gonadal steroids at puberty do not accrue bone normally and men who become hypogonadal in adulthood develop high-turnover bone loss. Gonadal steroid replacement in hypogonadal men (largely with aromatizable androgens) suppresses bone resorption and, in pharmacological doses, stimulates bone formation. Further, in most groups of hypogonadal men, androgen therapy increases bone mineral density.

Nonetheless, several questions remain. At the basic level, while advances have been made in characterizing the mechanisms involved in estrogen deficiency bone loss, the cellular mechanisms underlying androgen deficiency bone loss are less well defined. At the physiological level, the relative roles of androgens and estrogens in male bone metabolism are still unclear. And clinically, the level of gonadal steroid suppression necessary to trigger bone loss in men remains unknown. Finally, the effects of gonadal steroid replacement in elderly men with borderline hypogonadism remains controversial. Currently, there are no clearly defined, evidence-based treatment guidelines to help manage individual patients. Androgens and estrogens are potent agents that affect a wide variety of tissues in men. Their potential effects, both positive and negative, will require longer-term studies to be defined. Thus, until more robust and long term data are published, the use of gonadal steroids to increase BMD in men must always be evaluated in light of the potential systemic effects of these hormones, as well as the specific characteristics of the individual patient.

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Basic Aspects of Calcitonin in Skeletal Health

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ENDOGENOUS CALCITONIN

Calcitonin (CT) is a 32-aminoacid peptide that is secreted by thyroidal C cells in mammals and by the ultimobranchial gland in submammals. CT, that was originally identified as a hypocalcemic factor present in bovine serum (1) exerts its hypocalcemic effects primarily by directly inhibiting osteoclastic bone resorption (2).

Biochemistry and Molecular Biology

BIOCHEMICAL STRUCTURE OF CT IN DIFFERENT SPECIES

Structures of CT have been determined in many species, including man. All calcitonins (CTs) have 32 amino acids with a proline amide at the C-terminus and cysteine at the N-terminus that forms a 1–7 disulfide ring with another cysteine at position 7. Considerable homology in amino acid sequences exists within the ring structure between CTs from different species. Significant differences in the amino acid sequence from position 8 to 31 occur between unrelated species (3), however, the sequences in related species, such as ovine, bovine, and porcine, are quite similar. Human and rat CTs differ by only two amino acids (Fig. 1). Common features include a 1-7-amino-terminal disulfide bridge, a glycine at residue 28, and a carboxy-terminal proline-amide residue. Five of the nine amino-terminal residues are identical in all CT species.

The biological activities of the various CTs, measured by the hypocalcemic response in rats, differ. Studies of the structure-activity relationship have shown that the loss of only one amino acid results in the loss of almost all activity. However, the disulfide bond forming the ring structure has been replaced in eel CT with a C-C linkage without a loss of activity. Unlike that for parathyroid hormone (PTH), a biological active fragment of CT has not been discovered.

From the evolutionary point of view, changes in the amino acid sequence of CT from fish to mammals have occurred, but without changes in the number of amino acids. The

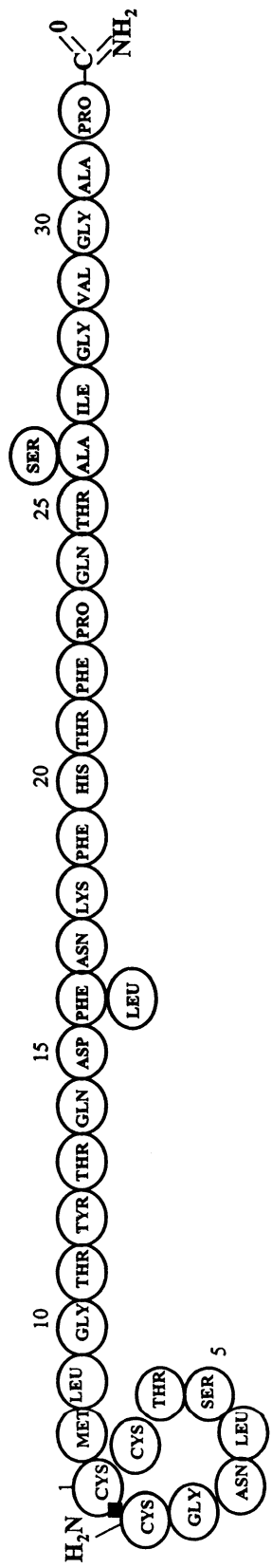


Fig. 1. The amino acid sequence of human CT, as compared to that of the rat when LEU and SER replace PHE and ALA at positions 16 and 26, respectively.

finding that receptors for CT, PTH, and many other polypeptide hormones have substantial homologies, indicates that these receptors may have evolved from a common ancestral molecule (4). The nonmammalian CTs have the most potency, even in mammalian systems. CTs of the nonmammalian vertebrates, in particular those of fish such as salmon and eel, have been reported to exert a greater hypocalcemic effect than mammalian CTs. Salmon CT (sCT) is the most active molecule in humans and is the most widely used for therapeutic purposes in the treatment of bone disorders, such as Paget's disease, hypercalcemia, and osteoporosis.

THE CALCITONIN FAMILY OF PEPTIDES

CT is related in sequence to other bioactive peptides, calcitonin gene related peptide (CGRP) 1 and 2, adrenomedullin and amylin (5). Except for CT and CGRP 1, each of these peptides derives from a separate gene and exerts characteristic actions at different targets. CGRP 1 and 2 are potent vasodilators and immunomodulators, with actions in the CNS. Adrenomedullin is also a potent vasodilator with some CNS actions. The actions of amylin are related to carbohydrate metabolism, to gastric emptying, and to CNS function.

CGRP and amylin are homologous 37-amino-acid peptides, the genes for which have a common ancestral origin (5) (Fig. 2). CGRP 1 is generated by alternative processing of mRNA from CT gene, located on the short arm of chromosome 11 in the locus 11p. The second form of CGRP, CGRP 2, differs from CGRP 1 by only 3 amino acids in humans and 1 amino acid in rats. It is produced by a separate gene also on the short arm of chromosome 11. These peptides have in common a 6-amino acid ring structure at the amino-terminus created by a disulfide bond between positions 2 and 7; the carboxy terminus is amidated. Amylin has 43% sequence identity with CGRP 1 and 49% with CGRP 2.

Despite their distinct bioactivities, this family of peptides shows some cross-reactivity at each other's receptor (5). There are separated specific receptors for amylin and CGRP, with some evidence for more that one class of receptor for CGRP (6). Amylin, CGRP, and CT displace each other from specific binding sites, indicating a significant crossreactivity of each with the receptors of the other peptides. Amylin and CGRP have calcitonin-like effects probably mediated by the calcitonin receptor. Recently, these peptides have been found to stimulate osteoblast growth, presumably via a different receptor since the CT receptor is not found in osteoblasts (7).

MOLECULAR BIOLOGY OF THE CT GENE

The CT gene consists of six exons separated by five introns. Two distinct mature messenger RNAs (mRNAs) are generated from differential splicing of the exon regions: one translates as a 141-residue CT precursor and the other as a 128-residue precursor for CGRP1. CT is the major post translationally processed peptide in thyroidal C cells, whereas CGRP-1, is the major processed peptide in neurons. The second CGRP, CGRP-2, is produced by a different gene. The CT/CGRP gene is one of the earliest studied examples of alternative RNA processing (8,9). The regulatory mechanism controlling this event is poorly understood. This gene is transcribed in two tissue types, the thyroidal C cell and specific neural cell types. In C cells, 95% of the CT/CGRP pre-mRNA is processed to include exon 4; this leads to production of CT peptide. In contrast, in neuronal cells, 99% of the CT/CGRP pre-mRNA is processed to exclude exon 4, resulting in inclusion of exons 5 and 6; this leads to the production of CGRP peptide. Interestingly, in malignant C cells, both CT and CGRP are produced in equal amounts, resulting from changes in splicing pathways (10).

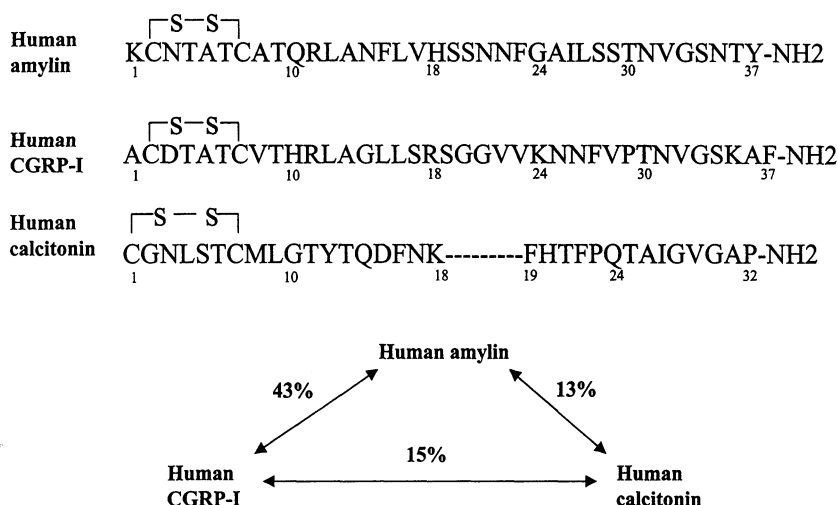


Fig. 2. Comparison of amino acid sequences of human amylin, CGRP and calcitonin. Amylin has 43% sequence identity with CGRP1, and 13% with CT.

BIOSYNTHESIS AND FUNCTION OF CT AND CGRP

Parafollicular thyroidal C cells are the primary source of CT in mammals, and the ultimobranchial gland is the primary source in submammals. C cells are neural crest derivatives, that produce also CGRP. Other tissue sources of CT have been described, including the pituitary cells and the neuroendocrine cells distributed in different tissues. The nonthyroidal sources of CT are not likely to contribute to its peripheral concentration, except in the case of malignant transformation, than can occur in ectopic (small-cell lung cancer) or eutopic cells (medullary thyroid carcinoma). In these tumors the peptide becomes a tumor marker. The mRNAs encoding the calcitonin gene transcripts includes precursors of CT and CGRP. It has been shown that human CT is derived from a large precursor that contains a calcitonin carboxyl-adjacent peptide (CCAP, also known as katecalcine), which has no hypocalcemic effect and is present in healthy subjects (11).

CT, with its variable and fluctuating serum levels in healthy people, has a complex physiological role which is still not well understood. CT is an endogenous regulator of calcium homeostasis, acting principally on bone. The hormone may have several roles in the regulation of many different functions involving calcium, including cellular permeability, neuromuscular excitability, muscle contraction, the activation of certain enzymes, endocrine secretion, cardiac function and blood coagulation. CGRP is produced in certain central and peripheral sensory neurons via alternative-processing of the primary CT gene transcript. CGRP is found mainly in the central nervous system; CGRP concentrations have been reported to be high in the spinal cord, amygdala and ventral striatum of the rat brain. In the spinal cord CGRP is found at the same sites as substance P, suggesting a possible role in nociception (12). In man CGRP is distributed throughout the CNS (neocortex cerebral cortex, periventricular mesencephalic region) with the highest concentrations in the posterior grey matter columns of the spinal cord and in the pituitary (13). Recent studies advance the concept that CGRP serves an important neuroeffector role in human epidermis (14) and in striated muscle fibers (15). Another neurogenic role for CGRP has been identified in vascular smooth muscle, where it acts as a rapid and potent vasodilator (16,17). CGRP is also present in skeletal afferent nerve

endings. In bone, this peptide may exert skeletal and mineral metabolic actions that may be identical to that of CT. However, pharmacological doses of CGRP are required to reproduce the osteoclastic cell response to CT, and some studies indicate that this effect of CGRP is mediated by cross-reaction with the CT receptors with a 500-fold lower biopotency (18). Recently, CGRP has been shown to stimulate the formation of osteogenic cell colonies from cultured rat bone marrow stromal cells (19).

Like CT, the physiological importance of CGRP is not yet elucidated. The main biologic effect of CGRP is vasodilation, but it also acts as a neurotransmitter and reacts with the CT receptors. The role of CGRP in bone is unknown, but it may be produced locally in skeletal tissue.

CT Receptors and Physiological Function

CALCITONIN RECEPTORS

CT has a large spectrum of biological and pharmacological activities. The actions of CT are mediated by high affinity CT receptors (CTR), that are widely distributed in multiple different tissues, including bone, kidney, CNS, placenta, ovary, testis, and lymphocytes. In bone, osteoclasts express several million receptors per cell. Receptors have also been detected in cell lines derived from tumors.

Initially, radioligand binding studies using iodinated CT and CT analogs, demonstrated that CT receptors were heterogeneous with respect to binding kinetics, with at least three distinct CTR subtypes (20). The recent cloning and characterization of CT receptors has given direct evidence for the existence of CTR heterogeneity and has provided information concerning the structural features of the CTR. Analysis of the amino acid sequence reveals an unexpected relationship of the CTR to other receptors. CTR belongs to a subfamily of G protein-coupled receptors with seven spanning domains, that includes receptors for PTH/PTH-related peptide, and for the secretin/glucagon peptide family (4). The similarities in the amino acid sequences and molecular structure of this subfamily of G protein-coupled receptors, suggest that these receptors were derived from a common ancestral gene. The first CTR was identified in porcine kidney. Although this porcine CTR is highly conserved among species, other CTR isoforms have been reported in other species, including rat, mouse, rabbit, and guinea pig. Multiple structurally distinct CTR isoforms, that possess functional distinct properties, have been cloned from human tissues or cells (21,22). The first isoform of the human CTR was initially isolated from an ovarian carcinoma cell line (BIN-67) (23) and later from other tissues (Fig. 3). The second isoform of the human CTR was isolated from a mammary carcinoma cell line (T47D) (24) and later from other cell lines and tissues (Fig. 3). Two different isoforms have been also described in human giant cell tumor of bone (21). The first isoform, designed as GC-10, differs from the CTR found in an ovarian carcinoma cell line, in the 5'-region in that it lacks a 71-bp segment. The second isoform, indicated as GC-2, lacks this 71 bp insert but also 48 nucleotides encoding part of the intracellular domain. In murine tissues, at least two different CTR isoforms are expressed: C1a, which corresponds to the common isoform conserved among species, and C1b, containing a 37-amino acid insert in the first extracellular loop. Although the C1b isoform was originally identified in rat brain and was shown to be relatively specific to brain, its expression in rat and mouse osteoclasts has also been demonstrated (20). These isoforms exhibit significant differences in ligand binding specificities and patterns of coupling to signal transduction pathways. The selective expression of individual receptor isoforms pro-

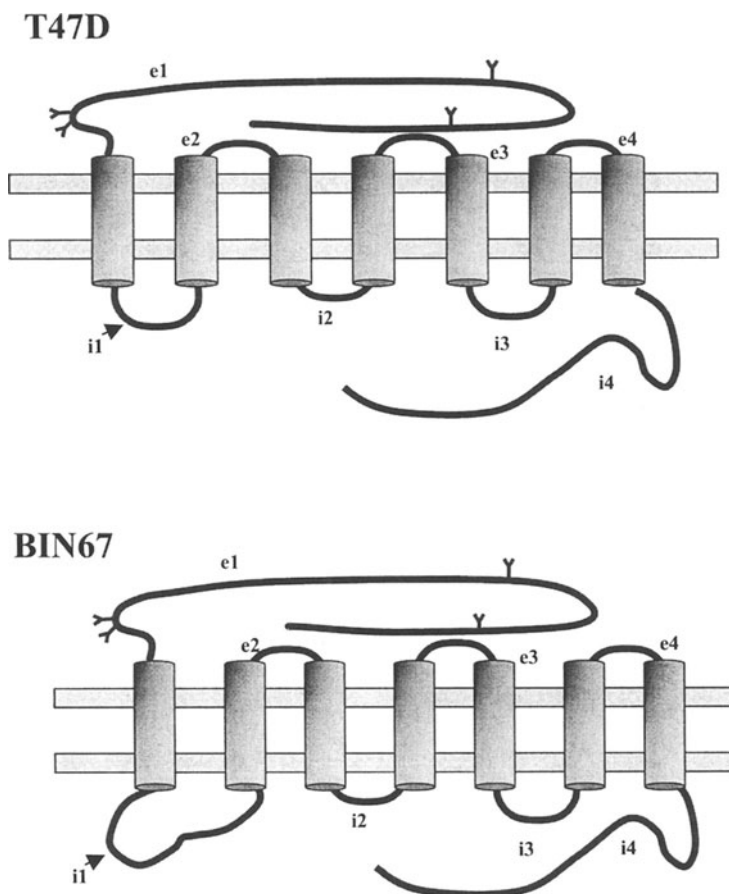


Fig. 3. Isoforms of human calcitonin receptors showing the extracellular domains (e1–e4) and intracellular domains (i1 to i4). In the human CTR cloned from BIN67 ovarian carcinoma cells (23), i1 contains a 16-amino acid insert which is not present in the CTR cloned from T47D breast cancer cells (24).

vides an important mechanism for producing tissue- or cell-specific responses to CT. Cells expressing the Ca1 receptor bind human and salmon CT with high affinity and demonstrate increases in cAMP levels after treatment with either ligand. Cells expressing the Cb1 isoform do not bind human CT and show minimal cAMP response after incubation with rat or human CT. Probably C1b isoform may function as a receptors for a ligand that is structurally related to salmon CT.

The structural analysis of human CTR from human blood samples has demonstrated the presence of allelic variants in the intracellular domain 4, expressing either proline or leucine as the 463rd amino acid. Among the Japanese population it was found that the variant with proline was the most prevalent type (25). The relationship between this polymorphism and bone mineral density has been studied in French and Italian populations. In Italy, it has been reported that individuals homozygous for the leucine substitution had reduced bone mass when compared with heterozygotes and proline homozygotes (26,27) (Fig. 4). In France, an association between bone mineral density and this polymorphism has also been found, showing that heterozygotes had higher bone

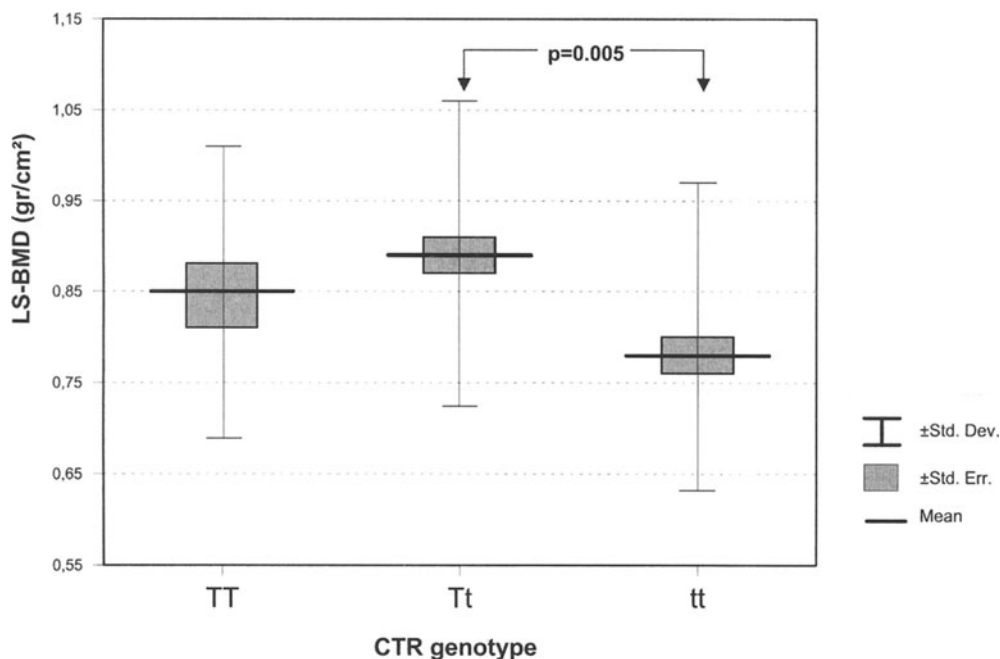


Fig. 4. Lumbar spine BMD values according to CTR genotypes. Subjects with tt genotypes showed significantly lower lumbar BMD in comparison with Tt genotypes ($p = 0.005$). Reproduced with permission from ref. 27.

mineral density and a reduced risk of fracture when compared with homozygotes (28). These findings suggest that the CTR gene could contribute to bone mass determination with other polymorphic genes.

ROLE OF CT IN CALCIUM METABOLISM

The exact physiologic role of CT in calcium homeostasis and skeletal metabolism has not been fully elucidated in humans. The principal role of CT is in the regulation of calcium metabolism to prevent calcium depletion or excess. CT directly or indirectly also controls the movement of other ions, such as phosphate and magnesium, in order to maintain the ionic equilibrium. CT, acting as a hormone of mineral regulation, exerts an effect at many levels: in bone, in the kidney and in the gastrointestinal tract.

In vitro experiments have demonstrated that CT inhibits PTH-induced osteoclastic resorption of bone in tissue culture inducing changes in the shape of osteoclasts and inhibiting their motility (29). CT also was reported to inhibit osteocytes and stimulate osteoblasts, but these effects are controversial. The reduction of osteoclastic bone resorption lowers plasma calcium. It has been postulated that the role of CT in the control of osteoclasts and other bony cells is to regulate the movement of calcium between the extracellular, intracellular and mitochondrial compartments. CT is claimed to enhance the accumulation into the mitochondria, while PTH, on the other hand, is reported to indirectly promote calcium efflux from the mitochondria.

At the cellular level, CT inhibits the cell's motile and secretory functions of osteoclasts, such as the processes of margin ruffling, granule movement, enzyme release and

proton secretion. CT arrests the continuous cytoplasmic motility. This is followed by cytoplasmic retraction (30). The phase of quiescence is rapid (half-time about 15 min) whereas the phase of retraction develops slowly (half-time about 27 min). These two effects of CT are produced by the activation of protein kinases A and C. The action of CT on the osteoclast involves the activation of a large number of high affinity surface receptors. The transduction mechanism for CT action on the osteoclast implicates the activation of the cyclic AMP system. CT directly elevates osteoclast cyclic AMP levels (31). The signalling mechanism also involves a rise in cytosolic calcium (32). Protein kinase C-induced phosphorylation may play a role in coupling the cytosolic calcium response with effector systems (33). The rise of intracellular calcium results from the release of intracellularly stored calcium via the generation of inositol 1,4,5-triphosphate (IP3) and the activation of a receptor-operated calcium channel (34).

In the kidney CT appears to play a minor role in renal function, being involved in electrolyte and water excretion, and consequently in calcium homeostasis. An increase in calcium, phosphate, sodium and water urinary excretion has been reported as a direct effect of CT on kidney receptors, but these effects occur at concentrations of the hormone that are supraphysiologic.

In the gastrointestinal tract CT seems to indirectly affect the function of both endocrine and exocrine glands, probably as a function of food ingestion. Several hormones of the gastrointestinal tract are potent CT secretagogues. CT inhibits the secretory activity of the stomach and pancreas, and increases the secretion of water, sodium and other ions in the gut, but these effects are associated with supraphysiologic concentration of the hormone.

DOWNREGULATION OF CTR

Molecular cloning of the CTR has made it possible to investigate the regulatory mechanism of CTR expression at the messenger RNA (mRNA) level. Recent studies have revealed that treatment with CT causes a decrease in the steady state level of CTRmRNA, and thus significantly contributes to homologous desensitization in osteoclasts (35–38). These studies have not resolved the issue regarding the mechanism of CTRmRNA downregulation by CT, including whether the regulation occurs at the transcriptional level. More recently, evidence has been given that CT downregulates C1a-CTRmRNA expression at least in part by a transcriptional mechanism in cells of the osteoclast lineage (39). This downregulation is independent of *de novo* protein synthesis and is thus most likely mediated by posttranslational modifications by a mechanism that involves the cAMP/protein kinase A pathway (39).

Secretion of Calcitonin

REGULATORS OF CT SECRETION

Calcium concentration in blood is the most important regulator of CT secretion. When blood calcium increases acutely, there is a proportional increase in CT secretion, and an acute decrease in blood calcium produces a corresponding decrease in plasma CT. However, the effects of chronic hypercalcemia and chronic hypocalcemia are not fully defined. It seems likely that C cells respond to sustained hypercalcemia by increasing CT secretion, but if the hypercalcemia is prolonged, the C cells probably exhaust their secretory reserve (40). The inhibitory effect on CT secretion by chronic hypocalcemia is more difficult to demonstrate.

Gastrointestinal peptides, especially those of the gastrin-cholecystokinin family, are potent CT secretagogues when given parenterally in supraphysiologic concentrations. However, it is possible that gastrin or related GI hormones, may act as the natural secretagogues that increase CT after oral ingestion of calcium or other food components (41).

Many other neuroendocrine and ionic factors have been shown to regulate CT secretion under experimental conditions, but it is unlikely that these agents participate in the physiologic regulation of CT secretion. Probably the blood levels of some hormones may influence CT secretion in particular physiologic situations, such as during growth, pregnancy and lactation. A rise in the blood levels of the vitamin D metabolite $1,25(\text{OH})_2\text{D}_3$ increases CT secretion during pregnancy or lactation, probably as a defense mechanism to protect the maternal skeleton (42). Estrogens increase blood levels of CT, suggesting that in surgical and natural menopause oestrogen deficiency may be a factor in the pathogenesis of osteoporosis with low CT levels (43).

BLOOD LEVELS AND METABOLISM OF CT

The normal basal blood levels of immunoreactive endogenous CT are in the range of 30–90 pg/mL (44). The quantity of CT secreted daily is reported as 50–250 μg , corresponding to 10–50 International Units (IU) (45).

In humans, the basal level of CT has been found to be high at birth and during infancy and to decrease gradually with age, with levels that appear to be lower in women than in men (46) (Fig. 5).

The circulating CT is derived from a precursor. CT is present in the blood in several forms, with high molecular weight, most of which are probably inactive. The only biologically active form is a monomer (which is identical to synthetic human CT) that is broken down into inactive fragments in the peripheral blood.

The metabolism of CT involves many organs. Evidence has been given for degradation of CT by kidney, liver, bone and thyroid gland. The kidney seems to be the most important organ of clearance of the hormone.

Clinical Abnormalities of CT Secretion

In man C cells are present in the lungs, in the thymus as well as in the thyroid (47,48). This explains why CT has been found in the blood of patients after thyroidectomy.

The pathological conditions that are associated with depressed CT blood levels are not well defined. Low levels of immunoreactive CT have been described in osteoporosis following ovariectomy or natural menopause (49), in osteoporosis secondary to hypothyroidism (50) or hypogonadism (51), in non-goitrous congenital cretinism (52) and in thyroidectomized patients.

The most common pathological condition associated with raised CT blood levels is the medullary carcinoma of the thyroid (MCT), that is the tumor of the parafollicular C-cells.

MEDULLARY THYROID CARCINOMA (MCT)

MCT is a malignant and highly metastatic tumor that secretes excessive amounts of calcitonin. MCT is rare, but may be present in more than 90% of individuals with multiple endocrine neoplasia (MEN) type IIA or IIB. In these cases MCT is associated with pheochromocytoma (MEN IIA and IIB) and hyperplasia of the parathyroid glands (MEN IIA). The CT blood concentration is an excellent marker for diagnosing MCT, that

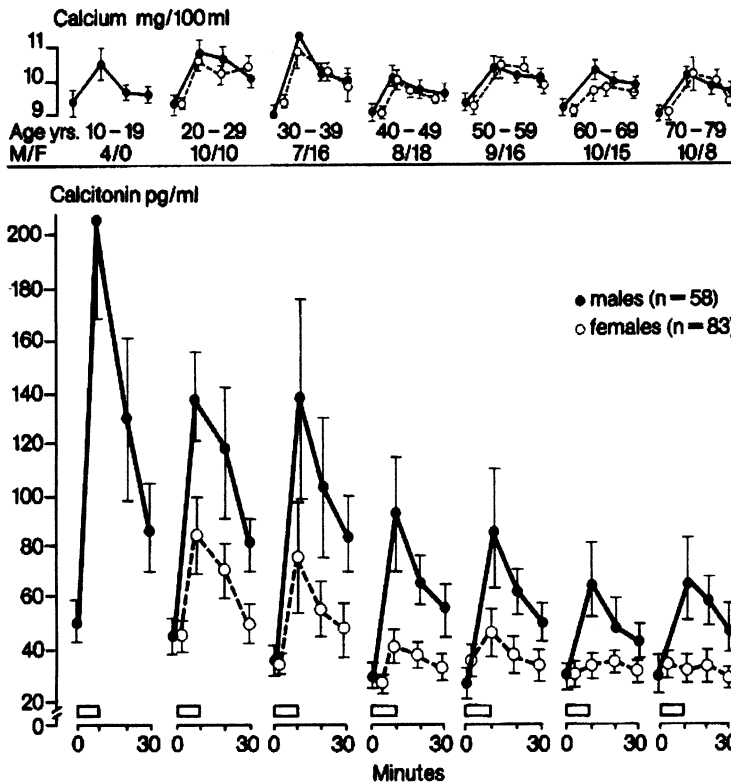


Fig. 5. Response of serum calcium and plasma calcitonin to 10-min infusion of calcium in 58 normal men (solid circles) and 83 normal women (open circles). Plasma CT is higher in men than in women ($p < 0.05$ – 0.001) and there is a progressive decrease of the response with age in both sexes. Reproduced with permission from ref. 46.

may cause blood levels as much as 5000 \times higher than normal. The plasma level falls to below 100 pg/mL during true remission, but remains elevated after incomplete excision of the tumor or when metastases have formed. When the blood levels of CT are normal or only slightly elevated, the best way of detecting the MCT is to use one of the dynamic tests based on provocation by calcium, ethanol, or pentagastrin (53–55). Plasma levels of CGRP are usually elevated in MCT (56) and it is quite possible that the episodes of flushing which occur in this disease are due to this peptide. MCT is curable in early stages by removal of the thyroid before the appearance of metastases to local lymph nodes, bone, and other tissues.

Although MCT is the source of the most profuse CT secretion, CT is also secreted by other types of neoplasms. These CT-secreting tumors may be extrathyroid, such as breast cancer or small-cell carcinoma of the lung, or originated in tissues containing C cells, such as tissue derived from the neural crest or tissue belonging to the amine precursor uptake decarboxylation (APUD) system (57). The CT secreted by such tumors may be normal or abnormal in structure. However, immunoreactive CT may be a useful marker for diagnosis and for monitoring the effectiveness of treatment in such cases (58–60).

CALCITONIN AS A DRUG

Natural porcine calcitonin and synthetic human, salmon and eel analog calcitonins are the forms of the hormone employed in the therapeutic use, their principal indication

being bone diseases characterized by excessive bone resorption and skeletal loss such as hypercalcemic states, Paget's disease of bone and osteoporosis. The potency of mammalian CTs is estimated between 100–400 IU/mg peptide, whereas the non mammalian CTs show higher potencies, around 2000–6000 IU/mg peptide. The hypocalcemic effect is higher and more prolonged for salmon calcitonin (sCT) than for human calcitonin (hCT) (61,62). sCT is approx 40–50 times more potent than hCT. Therefore, sCT is the most active molecule in humans and is the most widely used for therapeutic purposes.

CTs have to be given parenterally because they are hydrolyzed and inactivated in the gastrointestinal tract. Traditionally, CTs have been administered by subcutaneous and intramuscular injection or, less often, by intravenous injection. More recently, the development of a non invasive route of administration, with the nasal spray formulation of sCT, has offered a significant therapeutic advance that avoids the discomfort of repeated injections.

Pharmacokinetic Properties of sCT

PHARMACOKINETIC CHARACTERISTICS OF sCT GIVEN PARENTERALLY

The methods of assay and the routes of administration are the two main factors that influence the pharmacokinetic properties of sCT. The two principal methods are measurements of radioactivity after administration of a tracer dose of sCT labelled with radioactive iodine, and measurements by a radioimmunoassay (RIA) specific for sCT. The two methods have some limitations. For the first method, the measurement of radioactivity does not confirm that the radioactive label is still attached to the peptide. For the RIA method, the concentrations of immunoreactive sCT without or following extraction, are not necessarily the same as those of biologically active sCT. In addition, the sensitivity of the assay method (limit of detection) is an important factor because of the relatively low concentrations at which the hormone is biologically active. However, RIA is the technique most often used (63,64).

sCT is efficiently absorbed after parenteral injection, the half-life of absorption is 20–25 min, and the peak plasma concentration is attained in less than 30 minutes. The half-life of elimination from plasma is 70–90 min and the metabolic clearance rate around 200 mL/min. Plasma protein binding is 30–40%. The apparent distribution volume after subcutaneous injection is about 20 L. The absolute bioavailability after parenteral injection (subcutaneous or intramuscular) is about 70%. The principal site of degradation of sCT is the kidney. Only about 2% of the dose is excreted as unchanged immunoreactive sCT in the urine.

PHARMACOKINETIC CHARACTERISTICS OF sCT GIVEN BY NASAL SPRAY

Following intranasal administration of sCT, peak plasma concentrations are achieved in 30–40 min, which is slightly longer than the time of peak reached after parenteral administration (15–20 min) (65–67). In general, the peak plasma concentration values are lower, but more sustained after intranasal than after parenteral administration of sCT (65,68,69). Elimination half-life of intranasal sCT has been calculated to be 43 min. A dose-dependent increase in plasma concentrations has been shown following single-dose intranasal administration of sCT 50, 100, and 200 IU (65–67). In healthy volunteers, the bioavailability of intranasal sCT is approx 3% compared with the same dose administered by parenteral injection. However, evaluations of the acute biochemical response to sCT indicate that intranasal administration provides approx 25–50% of the biological activity of the same dose administered parenterally (65,70,71).

Pharmacodynamic Properties of sCT

BIOCHEMICAL RESPONSE

The principal pharmacodynamic effects of a parenteral dose of CT on blood chemistry in man are hypocalcemia and hypophosphatemia. These effects are mainly due to the antiosteolytic action of CT, but also to its action on the kidney. In man the hypocalcemic effect may last for several hours, reaching a peak between 3 and 7 h after administration (62). The magnitude of the effect depends on the dosage of the hormone and on the intrinsic potency of CT. In healthy volunteers 50 IU of sCT appear to be equipotent with 75–90 IU of hCT (62). The effect also depends on the level of bone remodeling activity. In patients with Paget's disease of bone the hypocalcemic effect for the same dosage of CT is greater than in normal subjects. In these patients sCT appears to be about ten times more potent than hCT (61). The hypophosphatemic effect of CT, which is the result of the lower rate of bone resorption combined with the hyperphosphaturic effect, increases with the dose.

In healthy volunteers CT induces a significant rise in serum levels of cyclic adenosine monophosphate (cAMP) as a result of activation of the adenylate cyclase-cAMP system when the peptide interacts with CT receptors on osteoclasts, with a peak of circulating cAMP at 60 min and a return to the baseline value at 240 min (62) (Fig. 6). sCT seems to have a greater effect on cAMP levels than hCT (Fig. 6). This effect appears to be dose-dependent.

Studies in which sCT 50–400 IU was administered intranasally as a single dose to healthy volunteers or postmenopausal women showed similar short-term effect such as reduced serum calcium and increased serum cAMP levels (65,68,72). Higher doses of intranasal sCT (200–400 IU) were typically associated with significant reductions in serum level of calcium and phosphate which lasted up to 6 hours, and with significant increases in serum cAMP levels. Comparative studies evaluating the effects of intranasal and parenteral sCT in healthy volunteers demonstrated equivalent biological effects when the intranasal dosage was approx 2–4 times that of the parenteral dosage (65,70,73).

BIOCHEMICAL MARKERS OF CT ACTIVITY ON BONE

A number of blood and urine chemistry variables which act as markers for bone cell activity and bone diseases, exhibit changes after CT administration. In healthy volunteers parenterally administered sCT has been associated with a reduced urinary excretion of hydroxyproline and pyridinium crosslinks, that are markers of bone resorption (74). During recent years, new specific biochemical markers for both bone resorption and formation have been developed and have made it possible to investigate the dynamic effect of therapy and the optimum treatment regimen with CT. The serial measurements of biochemical markers of bone turnover (serum alkaline phosphatase, serum osteocalcin, fasting urinary hydroxyproline, pyridinoline, deoxypyridinoline, and crosslaps) in postmenopausal women treated with nasal sCT showed maximum decreases of 10–23% between 6 and 9 mo, after which an escape from therapeutic effect was observed (75). In a study in which selected postmenopausal women with significantly accelerated bone turnover were chosen for treatment with intranasal sCT, the hormone effectively reduced bone turnover, as assessed by biochemical markers, the maximum effect being reached after 8 wk of treatment (76). Thereafter, a plateau was reached and subsequently the biochemical markers of bone resorption showed a trend to increase. The gradual decrease of the skeletal response to CT with regard to bone turnover that has been observed in many studies with continuous treatment regimens indicates the development of a resistance to CT.

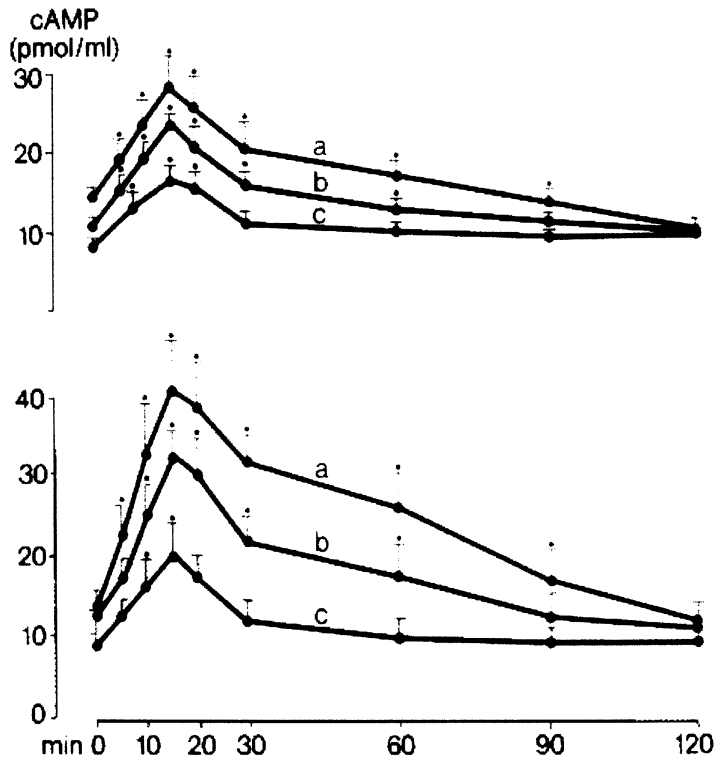


Fig. 6. Mean (\pm SEM) cAMP plasma levels in 10 healthy volunteers after 10-min infusion of salmon CT (a), human CT (b), and porcine CT (c), at two different dosages, 50 IU (upper panel) and 100 IU (lower panel). The dose-dependent effect on cAMP levels is greater with sCT than with hCT and pCT. Reproduced with permission from ref. 62.

RESISTANCE TO CT

The inhibitory effect of CT on bone resorption is not always sustained. This effect was reduced or disappeared after 12–48 h in cultures of bone tissue, treated with resorption stimulants, despite continuous addition of CT (77,78). The mechanism of this “escape phenomenon” is still unclear. Also, long-term continuous treatments with CT are associated with a progressive decrease in responsiveness. Possible explanations include secondary hyperparathyroidism, due to the hypocalcemic effect of the hormone, the formation of neutralizing antibodies to CT, and a reduction in the number of specific receptors (downregulation of CTR). In long-term treatment with CT a condition of secondary hyperparathyroidism has been rarely described. Although formation of antibodies against CT has been demonstrated in subjects treated with sCT, there is evidence that it does not affect the biological response. Some studies have confirmed the fact that a leveling off in the skeletal response to sCT develops independently of antibody formation (76,79). Probably, the decrease in responsiveness to CT in the long-term, revealing an escape from the therapeutic response, is mainly due to the downregulation of CTR (80). As downregulation is due to a reversible decrease of number and sensitivity of specific receptors following chronic exposure to CT, it can be expected that the responsiveness of the receptors is restored when the treatment is interrupted. Accordingly, the optimum treatment modality for CT should be discontinuous, in order to minimize the downregulation of receptors. In fact, some studies have shown that sCT is more effective when given intermittently rather than continuously (81,82).

Effects of CT on Animal and In Vitro Models of Skeletal Metabolism

In a recent review of the literature on the preclinical basis for the therapeutic use of CT, the effects of CT have been categorized as to the influence of the hormone on animal bone histomorphometry, bone mass, mechanical properties, experimental fracture healing, and osteoblastic activity (83). In a variety of bone loss histomorphometric models in rat and other animals, including excessive bone resorption induced by ovariectomy, immobilization and administration of cyclosporine, an inhibitory effect of CT on bone resorption rate was noted in the large majority of studies. The decrease in trabecular bone volume induced in these models was prevented half or more by the administration of CT. In studies performed on rat cortical bone histomorphometry the effect was quantitatively smaller but qualitatively similar to that observed on rat trabecular bone. CT protected against bone loss due to increased bone resorption not only the rat, but also the mouse, the hamster, the pig and the dog. Bone formation was variably affected, with increases in some studies, no change or decreases in others (83). Consonant with the histomorphometric effects in several different species, bone mineral density measured by a variety of methods, increased, reversing the bone loss induced by the model (83). Some studies addressed CT effects on mechanical properties of bone. In rabbit and rat bone strength was improved by sCT treatment for 12–25 wk (84,85). In the ewe, resistance to compression of the femur was significantly improved in a dose-dependent manner, using 50 and 100 IU doses of sCT (86). In other studies using human CT no benefit was observed.

Many studies have explored the effects of CT on experimental bone disruption, by surgically or traumatically induced fracture. In different species, no impairment of bone healing by CT administration was noted, and in some studies a shorter duration of healing was reported (83).

The studies on the effects of CT on bone formation and mineralization in animal models suggest an anabolic effect of the hormone, particularly on cartilage formation, bone matrix synthetic activity and bone growth (83). The effects of CT on bone quantity and quality are primarily a result of potent antiresorptive activity. Some histomorphometric studies have shown an inhibitory effect of the hormone on bone formation as a secondary phenomenon. However, other direct studies of the effects of CT on osteoblastic function suggest an independent anabolic effect. Recently, it has been suggested that CT stimulation of bone formation could be the result of cross-reactivity among bone cell receptors for CT, CGRP and amylin. Both in vitro and in vivo studies have shown significant increases in osteoblast-like cell number in fetal rat bone cell cultures and in histologic parameters of bone formation after calvarial injections in adult mice with these peptides (87). Amylin was most active in affecting bone formation, whereas CGRP was more similar to CT. Because both peptides, amylin and CGRP, circulate in very low concentrations, it is possible that CT stimulation of amylin and CGRP receptors may explain the anabolic effect of CT. More recently, evidence has been given that sCT inhibits apoptosis of murine osteocytic and osteoblastic cells, isolated from calvaria in vitro, most likely via actions mediated by receptors linked to the adenylate cyclase system (88). Prolongation of the life span of osteocytes and osteoblasts by sCT may explain the decrease in bone fragility and contribute to the antifracture efficacy of the hormone, which is disproportional to the relatively modest increase in BMD.

Effect of Calcitonin on the CNS

Immunoreactive CT has been identified in several organs and tissues not primarily related to calcium homeostasis, including the brain (89), cerebrospinal fluid (90), and

pituitary gland (91). The physiological role of the presence of CT in the CNS remains unclear. However, the demonstration of CT binding sites, distinct from CGRP binding sites, in rat and human brain and pituitary (13,92–94) has raised the possibility that the hormone may have some neuroactivity in the CNS.

In fact, both the CT peptide, or an analog, and CT receptors are found in areas of the CNS involved in the control of appetite, lactation, and pain perception, and both have also been found in the hypothalamus, suggesting that CT may have a neuromodulator function. In animals, direct CNS administration of CT by the intracerebroventricular route produces different effects, including reduced prolactin secretion, inhibition of gastric acid secretion, appetite suppression, as well as effects on some neurotransmitters and nociception (44).

THE ANALGESIC EFFECT OF CT

A central analgesic action of CT has been demonstrated in animals, while in humans it is effective in relieving bone pain associated with Paget's disease, osteoporosis and metastatic bone disease (95). The analgesic action of CT on bone pain due to skeletal disorders appears to be independent of its action on osteoclastic bone resorption, as pain relief precedes any change in biochemical indices of bone turnover (95). Several short-term, mostly uncontrolled studies have reported analgesic properties of CT in extraskelatal painful conditions of different nature. In some reports, wherein a placebo-controlled design was used, CT appeared to induce pain relief in some neurologic conditions. In patients with migraine, parenteral sCT was significantly more efficient than placebo in reducing the frequency of pain episodes (96,97). Likewise, CT was able to reduce the pain score in patients with the phantom limb pain syndrome in the early postoperative period (98,99), and in mild cases of lumbar spinal stenosis (100). The reported analgesic effect in all this conditions that are not associated with metabolic abnormalities of bone remodeling further indicates that this action of CT is not mediated by its effect on bone turnover. Reduction of pain has also been observed in metastatic tumors with bone localizations (101–104). The mechanism of CT-induced analgesia in these cases may also involve a decrease of bone resorption activity in the metastases, with reduction of bone erosion.

POSSIBLE MECHANISM OF ACTION OF CT-INDUCED ANALGESIA

The mechanism underlying CT-induced analgesia is open to speculation. A number of hypotheses have been advanced, including inhibition of prostaglandin and thromboxane synthesis, interference with calcium flux, involvement of the cholinergic or serotonergic systems, stimulation of β -endorphin release, and a direct action on CNS receptors (95,105). In man, similarities between CT- and morphine-induced analgesia (106,107) and reports of CT-induced elevation of plasma β -endorphin level (95,108–110) suggest the possible involvement of the endogenous opiate system in mediating the analgesic action of CT. Beta-endorphin is produced in the intermediate lobe of the pituitary by posttranslation processing of a precursor, proopiomelanocortin, common to ACTH and MSH (111). In studies reporting an increase in peripheral β -endorphin levels following parenteral injection of sCT, cosecretion of ACTH was also observed (95,108,109) (Fig. 7) suggesting a potential modulatory action of CT on the secretory activity of these pituitary cells (112). However, the evidence that the analgesic effect of CT is mediated by an interference with the endogenous opiate system remains tenuous.

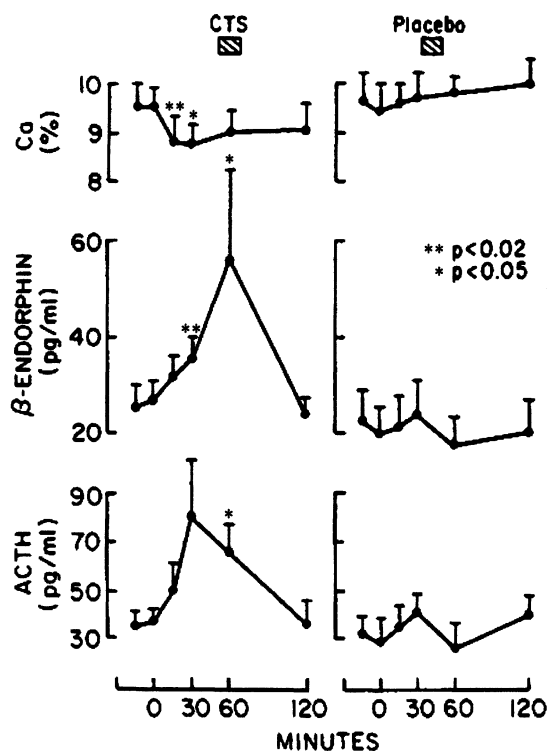


Fig. 7. Effects of placebo and salmon CT (CTS) infusion on plasma calcium (%Ca fall from baseline), circulating beta-endorphin material, and ACTH in five patients with malignant bone metastases and pain. Reproduced with permission from ref. 95.

The demonstration of CT binding sites in areas of the brain involved in pain perception and a series of animal studies have raised the possibility that CT may directly modulate nociception in the CNS (92). In support of this hypothesis are studies in animals showing direct effects of CT on serotonergic or monoaminergic pathways (113), on intracellular calcium levels in the CNS (114), and observations of an analgesic effect obtained by direct epidural or subarachnoid injection of CT in man (115,116).

Intracerebroventricular, but not subcutaneous, administration of sCT raises the pain threshold in rabbits (117), and this effect does not involve opiate receptors since it is not antagonized by naloxone, and appears to be sustained on repeated dosing (118). Probably, the most likely mechanism of the analgesic effect of CT is a direct action on specific receptors in the CNS. Nevertheless, if CT directly acts on the CNS it must cross the blood-brain barrier when given parenterally. How CT, a peptide hormone, can cross the blood-brain barrier is still unclear. A peripheral analgesic effect of CT has also been proposed, on the basis of an inhibition of thromboxane production by the hormone (119), and the report of an enhancement of pain threshold by locally injected CT in animals (120).

THE ANALGESIC EFFECT OF CT IN OSTEOPOROSIS

Most of the clinical studies aimed at defining the analgesic effect of CT have been performed in osteoporotic patients with acute vertebral fractures. Although pain from vertebral fractures is usually self-limiting and can be treated with simple analgesics and initial bedrest, many studies have reported measurable improvement of pain scores with

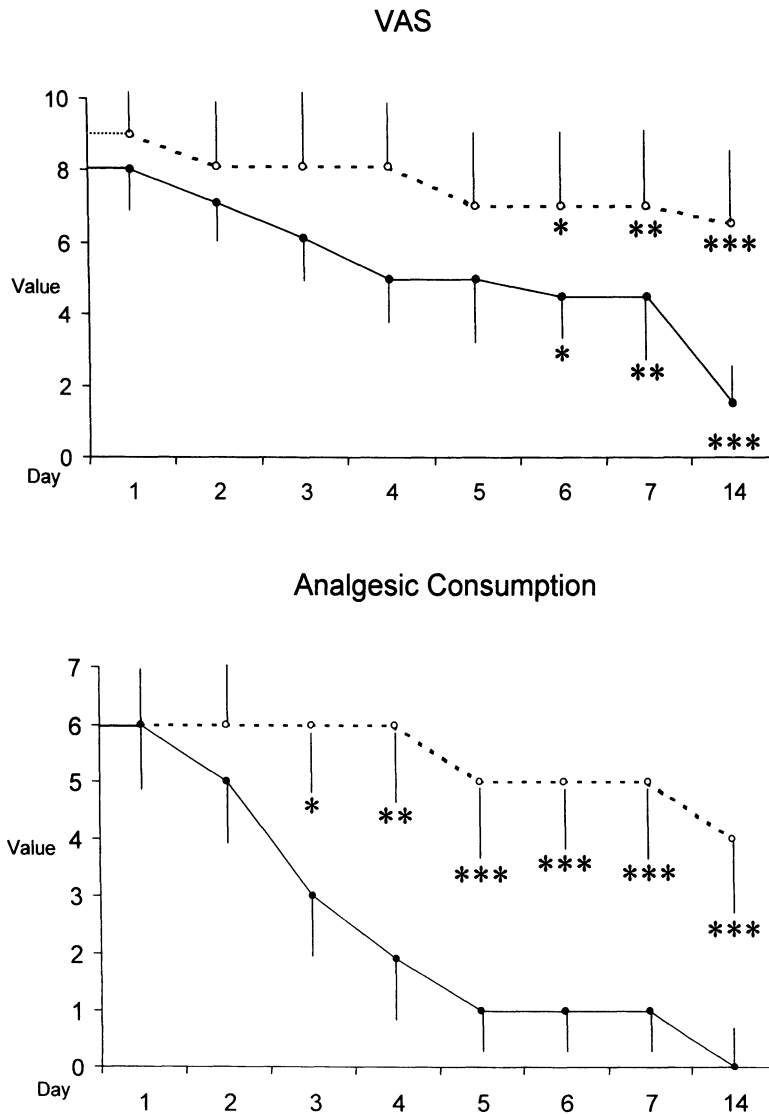


Fig. 8. Pain ratings according to a visual analog scale (upper panel), and consumption of paracetamol tablets (lower panel) in women after an acute vertebral fracture, treated with either intramuscular salmon CT (●) or placebo (○). Asterisks represent the significant difference between treated and controls at each time point by t test ($p < 0.05$ – 0.001). Reproduced with permission from ref. 122.

CT in subject with acute vertebral fractures, when the treatment was started within two weeks since the acute episode (121–123) (Fig. 8).

This effect was also associated with a decrease in the consumption of analgesic required by the patients to control pain and with an improved ability to sit, stand and walk (122,123). Although in all these studies a placebo effect, or a spontaneous partial resolution of the condition occurred, the decrease of the painful symptomatology was faster and more pronounced in these subjects taking CT (121–123). Analgesic effects have been obtained with doses of CT commonly used for treatment of osteoporosis, and with either parenteral or intranasal preparations.

In one controlled study comparing intranasal sCT 200 IU/d with intramuscular sCT 100 IU/d pain scores were significantly decreased by the second week with intranasal sCT, and only by the fourth week with intramuscular sCT (124). The notion that intranasal administration is more effective than parenteral injections in inducing pain relief remains to be confirmed. More recently, the analgesic efficacy of nasal spray (200 IU/d) and subcutaneous (50 IU/d) formulations of sCT has been evaluated in 204 patients with a recent, painful, vertebral crush fracture according to a double-blind, double placebo design (125). Relief was obtained in less than 10 d for more than 50% of patients, and the equivalence of the two formulations was demonstrated. Taken all together, these controlled studies provide evidence for a potentially useful extraskeletal action of the hormone, in addition to its antiresorptive action.

TOLERABILITY OF EXOGENOUS CT

Adverse reactions occur to some extent with all the CTs. Symptoms of various types are reported in all studies employing parenteral injections, including gastrointestinal (nausea, vomiting, abdominal pain, diarrhoea, unpleasant metallic taste in the mouth), vascular (facial flushing, sensation of facial warmth, tingling in the extremities), renal (polyuria, urinary urgency), and local (erythema and pain at the site of injection) symptoms (95).

An allergic reaction with a rash has been rarely described. These side-effects are inconvenient rather than serious. The severity of side-effects to CT is dose-dependent. In most cases reduction of the dose either attenuates or abolishes these effects (95). As expected, side-effects are much more severe when CT is given intramuscularly and minimized with subcutaneous injections, because of the higher blood CT peak obtained by intramuscular injections. Furthermore, tolerance appears to be different for each CT species.

Interestingly, some data suggest that human CT causes more adverse reactions than teleost CTs (salmon and eel), after administration of doses of equal biological activity (126,127). Adverse events when the hormone is given parenterally can be estimated to occur in approx 20–30% of treated patients. The most frequent complaints from injectable preparations are flushing and irritation at the injection site. Nausea was reported by about 15% of patients, and urinary symptoms occurred in 10–15% of the cases, whereas headache and vomiting were reported by less than 10% of patients (127).

Many of the side effects experienced with the injectable forms do not occur at all with the intranasal preparation, suggesting different absorption efficiencies and tissue distribution patterns for the two different administration routes. For the intranasal route, flushing was still the most frequent complaint (20%), followed by local symptoms, such as nasal congestion and irritation (16%), and rhinitis (8%) (128). Sporadic episodes of epistaxis and partial loss of sense of smell have been also reported. Although the pathogenesis of the systemic side effects is not clear, most vasomotor symptoms, including flushing and headache, may be related to an interaction of CT with receptors for CGRP (18), the hormone with potent vasoactive properties.

Finally, reactions to CT, which characteristically occur at the initiation of therapy, tend to either decrease or disappear with continued administration of the hormone.

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Clinical Utilization of Salmon Calcitonin in the Treatment of Osteoporosis

*Rationale, Supportive Data,
and Considerations for the Future*

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INTRODUCTION

Calcitonin, a physiologic endogenous inhibitor of bone resorption, decreases osteoclast formation (1,2), osteoclast attachment (2,3), and bone resorption in organ culture and in animal models (1,4,5). Thus there is a rationale for treatment with calcitonin, and specifically salmon calcitonin (S-CT), of diseases associated with increased bone resorption, such as osteoporosis. The last twenty years has witnessed an accumulation of clinical trials with S-CT providing supportive data for such a clinical indication. Also, the ongoing development of newer preparations of S-CT (an oral formulation), and the applicability of S-CT for newer indications (for pain, for hip fracture, etc.) via new clinical trials promises continued usage of this therapeutic agent for the future management of osteoporosis.

As salmon calcitonin is the principal species of calcitonin utilized clinically, the following discussions will deal primarily with this calcitonin preparation.

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BASIC PHARMACOLOGY OF SALMON CALCITONIN

Like all the known naturally occurring varieties of calcitonin, S-CT is a 32 amino-acid peptide with a free amino group on its N-terminal and a ring of seven residues formed by a disulfide bridge between two cysteine residues in positions 1 and 7 of the chain. It is of interest that, while S-CT appears to be one of the most potent varieties of the hormone in terms of its pharmacological effects in man, only 15 of its 32 amino acids are located in the same positions in the chain as in endogenous human calcitonin, while rat calcitonin has 30 out of 32 residues in common with human CT. Structure/activity relationships are thus still something of a mystery where the calcitonins are concerned.

Pharmacologically S-CT acts via specific calcitonin receptors, which binding studies have shown to be located in many different tissues (6–8). It exerts biologically relevant effects most notably in bone (9), the kidney (10), and the CNS (11).

The CT receptors on osteoclasts are particularly high-affinity receptors (12), which probably explains the primacy of CT's effect—and thus its therapeutic usefulness—in pathological conditions of bone associated with increased bone resorption. As a corollary to this, S-CT has been shown to be the variety of CT with the greatest affinity for all known types of calcitonin receptors (13).

The primary pharmacological effect exerted by S-CT on bone involves direct inhibition of the number, formation rate, motility and activity of osteoclasts. It may also regulate osteoblast activity (14) so that, overall, it may play a vital part in the bone remodeling/renewal process. Above all, its role is the prevention of excessive resorption, which it achieves specifically by inducing an acute quiescence of cell motility, followed by gradual retraction of the osteoclast's pseudopods (15–18), coupled with the formation of intracellular retraction fibers and a cessation of membrane ruffling, and thereby leaving a small, rounded, non-motile cell (19). Other mechanisms are also inhibited, such as the release of acid phosphatase (20), carbonic anhydrase II (21), focal adhesion kinase (22), and osteopontin (23), all of which are involved in various ways in the resorptive activity of the osteoclasts. In addition, it has recently been shown that S-CT does not induce early apoptosis of either osteoclasts (24), osteoblasts, or osteocytes (25). Prolongation of the lifespan of osteocytes in particular may be one reason for the beneficial effect of S-CT on bone strength and hence on fracture rate.

PREVIOUS CLINICAL TRIALS WITH SALMON CALCITONIN

A therapeutic rationale for S-CT in the treatment of osteoporosis therefore exists; what are the previous clinical trials confirming such a rationale? Before discussing such trials, it should be noted that the initial S-CT studies were performed with the parenterally (intramuscular or subcutaneous) administered form of S-CT; the past 15 yr have seen the nasal spray (NS-SCT) utilized for the majority of such studies. As well, as with other osteoporosis therapies, until recently (the past 5–7 yr) the clinical efficacy of NS-SCT was determined primarily by effects on bone quantity (total body calcium by neutron activation analysis: TBC-NAA, a measurement of total body bone mass; and bone mineral density [BMD] measurements with dual photon absorptiometry [DPA], single photon absorptiometry [SPA], or dual energy x-ray absorptiometry [DEXA]). An accepted tenet of such earlier studies was that changes in bone quantity could be extrapolated into effects, presumably beneficial, on skeletal fracture reduction.

An early study with parenterally administered S-CT in postmenopausal osteoporotic women with established disease demonstrated a beneficial increase in TBC (total skeletal bone mass) as measured by NAA through two years (26); subsequent studies with NS-SCT demonstrated significant increases in BMD at spine, hip, and wrist as determined by DPA, SPA, or DEXA over 1- to 3-yr periods (27–30). In 1992 Overgaard et al. (29) randomized 208 elderly women (between the ages of 68 and 72 yr all with low wrist BMD) to placebo nasal spray, or calcitonin for two years, as well as 500 mg of calcium daily. Women completing the trial in the 200 IU NS-SCT group ($n = 41$) demonstrated an increase of 3% in lumbar spine BMD by DEXA versus individuals completing the trial assigned to placebo and calcium. The latter group showed a mean increase of 1%. The treatment group was significantly different from the placebo group, but only at 6 mo ($p = <0.05$). A dose response was seen in lumbar spine BMD. In 1996 Ellerington et al. (30) demonstrated a significant increase in lumbar spine BMD by DEXA after 2 yr in women treated with 200 IU of NS-SCT; the women in this trial were either early or late postmenopausal; no calcium supplementation was given. In this trial as well women receiving intermittent (3 d/wk) NS-SCT at a dosage of 200 IU showed a nonsignificant increase in lumbar spine BMD.

Additional trials indicated beneficial effects of increases or preservation of BMD by NS-SCT in early postmenopausal women, i.e., a potential benefit for the prevention of osteoporosis (31,32).

One additional study indicated that withdrawal of S-CT was followed by immediate bone loss (33), suggesting the necessity of continued treatment to avoid an accelerated bone loss following therapy discontinuation.

An expected decrease in bone resorption with S-CT administration was originally shown with such markers of bone resorption as urinary hydroxyproline (34), and subsequently by studies with urinary n-telopeptide (35).

Although as noted there have been numerous trials assessing an effect of S-CT on improving bone quantity (typically BMD), throughout the development of S-CT for the treatment of osteoporosis there has been a paucity of studies designed to test its efficacy in reducing fracture, the most important endpoint of osteoporosis study trials. Previous studies with either injectable or NS-SCT indicating fracture reduction at spine or hip have been retrospective (36), or if prospective, have involved small numbers of patients (29,37). While such studies suggest a potential benefit of SCT in reducing osteoporotic fracture, a definitive prospective trial, adequately powered, has until recently been lacking.

DOES NASAL SPRAY SALMON CALCITONIN PREVENT FRACTURE? THE PROOF STUDY (38)

The PROOF trial (Prevent Recurrence of Osteoporotic Fracture) was designed to assess the efficacy of NS-SCT in the prevention of spinal vertebral fractures. One thousand, two hundred fifty-five postmenopausal osteoporotic women (mean age 68, 78% with previous fracture, and all with decreased BMD—a T score of -2 —at lumbar spine by DEXA) were recruited into a 5-yr double blind, controlled, randomized osteoporosis study. Women were randomized into placebo, 100, 200, or 400 IU dosages of NS-SCT, plus 1000 mg of oral calcium and 400 IU of vitamin D.

In the women with prevalent vertebral fracture, over the 5-yr study duration there was a significant 36% reduction in new vertebral fracture in the 200 IU dosage group, as compared to the placebo group (40 new fractures as compared to 60 new fractures, relative risk = .64 [0.43 – 0.96], $p = 0.03$). (Fig. 1). In the entire cohort including women

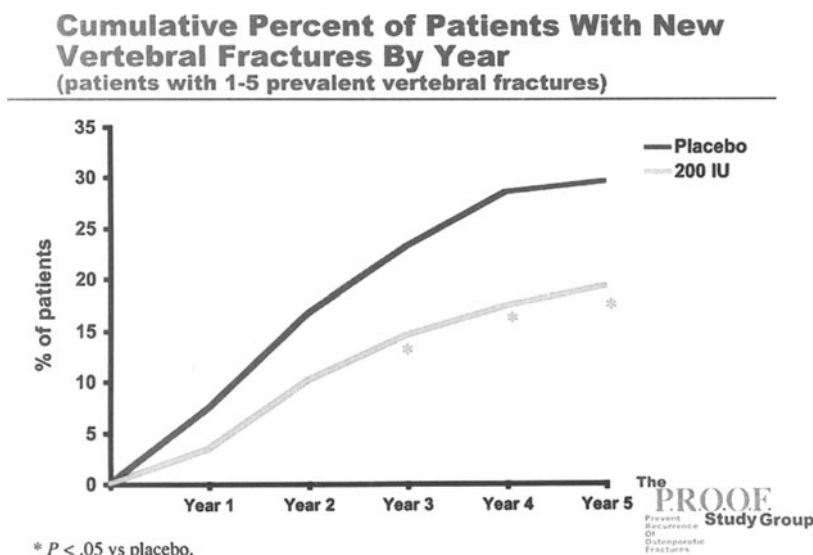


Fig. 1. Cumulative percent of patients with new vertebral fractures by year (patients with 1–5 prevalent vertebral fractures). * $p < 0.05$ (200 IU NS-SCT compared to placebo).

without prevalent vertebral fracture, the reduction in new compression fracture was a significant 33%. Other NS-SCT dosages including 400 IU did not demonstrate significant reduction in vertebral fracture. Eleven women needed to be treated for 3 yr with 200 IU NS-SCT to prevent 1 vertebral fracture.

This study was not designed or powered to examine the occurrence of hip fracture; nevertheless it is of interest that there were five new hip fractures in the 200 IU NS-SCT group, as compared to nine in the placebo group. The combination of the data from the 100 IU dosage group and 200 IU indicated a 68% reduction in hip fracture as compared to placebo; again however such a finding is a post hoc analysis in a study not designed to evaluate the occurrence of hip fracture.

BMD at the lumbar spine as determined by the DEXA technique increased significantly from baseline in all treatment groups through 5 yr (1–1.5%, $p < 0.01$). BMD was maintained at the hip. There was a significant decrease in the levels of bone resorption as determined by the serum C-telopeptide (CTX) in the 200 and 400 IU groups at 1 yr (about 25%, $p < 0.05$), with persistence of such effect through 5 yr.

The distribution of adverse effects was similar among the NS-SCT and placebo groups, except for a significant increase in rhinitis related to the study drug occurring in approx 20% of treated patients compared to 15% in placebo.

The effect of fracture reduction was persistent and sustained in the 200 IU group as compared to placebo through 5 yr (Fig. 1). No evidence of resistance or loss of effectiveness with increased duration of treatment was apparent in the PROOF trial.

From the PROOF trial it was concluded that 200 IU of NS-SCT significantly and safely reduces the risk of new spinal vertebral fractures, at a dosage of 200 IU as compared to placebo, in women at high risk for such fracture (i.e., a prevalent vertebral fracture at baseline).

A number of questions have been raised regarding the PROOF study; these include the dropout rate, the lack of a dose response, and the modest effects of NS-SCT on bone quantity (BMD) and bone turnover (bone markers—serum CTX). Each of these will be discussed.

First, 59% of study participants withdrew from the study prematurely. This dropout rate is not greater than that seen in other recently published clinical trials (39) in elderly, fragile, postmenopausal women. Contributing to the dropout rate was the fact that in this long term 5-yr trial it was not considered ethical to withhold BMD results from investigator and participant, which may have caused some participants to discontinue the study prematurely due to the approval of alendronate therapy in the United States during the calcitonin trial: i.e., the relatively modest increase in BMD (which participants and investigators may have perceived as a lack of efficacy) for NS-SCT as compared to a more robust increase with alendronate, with subsequent premature discontinuation. Also, analysis of baseline characteristics of participants at risk for a new vertebral compression fracture at 3 and 4 yr demonstrated that the dosage groups including placebo were well matched at those time periods, suggesting that selection bias did not occur. As well, participants who discontinued prematurely in the placebo group were losing bone at a greater rate than those who discontinued prematurely in the active calcitonin treatment groups, indicating a lack of bias due to poor responders to calcitonin in the calcitonin treatment groups discontinuing the study prematurely, while continuing in the placebo group.

Second, a dose response for the reduction in risk of new vertebral fracture was not demonstrated across dosages in this trial; there was however a significant biological effect of 400 IU NS-SCT on both bone markers (serum CTx) and BMD. Why these effects on these two components of osteoporotic fracture risk did not lead to significant reductions in the rate of vertebral fracture in the 400 IU group was not clear from the study; such a lack of a dose response was not felt however to negate the overall study conclusions.

Third, and perhaps most interestingly, NS-SCT reduced fracture risk without substantial effects on BMD, and with only modest effects on the serum CTx as a marker of bone resorption. Such findings are perhaps discordant from findings with alendronate (40) in which more robust effects on BMD and on markers was seen in association with vertebral fracture reduction. The PROOF study with NS-SCT is however similar to that of raloxifene (41) in which modest effects on BMD and markers of bone resorption were noted in association with vertebral fracture reduction. This has led to the evolving concept and hypothesis that therapeutic agents of a hormonal nature (NS-SCT and raloxifene as examples) may reduce fracture by different mechanisms than those of the more inert substances (such as bisphosphonates), with the former producing the majority of their antifracture effect through an effect on bone quality rather than on bone quantity, while the latter may exert their antifracture effect more on bone quantity and bone remodeling (42). This hypothesis is currently being tested in the QUEST trial as noted below.

QUANTITY VS QUALITY:

THE MECHANISM OF ACTION OF NS-SCT, THE QUEST STUDY

The PROOF study demonstration (39) of a modest effect of NS-SCT on BMD and markers, particularly as compared to bisphosphonates, in association with a significant reduction in vertebral fracture has raised questions regarding the relationships of bone quantity (BMD-DEXA), bone turnover/remodeling (particularly bone resorption), and bone quality (particularly microarchitecture). One study is currently underway to provide insight into this and other related issues. The Quantitative Effects Of Salmon-calcitonin Treatment (QUEST), a 2-yr, prospective placebo controlled clinical trial, is designed to determine the effects of NS-SCT on the primary endpoint of bone micro architecture. A number of methodologies with sequential assessments will compare the

characteristics of bone quality at multiple skeletal sites, as determined by iliac crest bone biopsy and subsequent histomorphometry, microcomputerized tomography of the biopsy specimen, and high resolution magnetic resonance imaging (43,44). QUEST is also expected to provide insight into interactions and interrelationships of bone quality, bone quantity (via DEXA and ultrasound), and BMD, and the biochemical markers of bone resorption and formation, as well as to evaluate the capacity of the various imaging methods utilized in the QUEST trial to define bone quality. The hypothesis being tested is that NS-SCT acts to reduce fracture by preserving trabecular struts and rods, particularly horizontal elements, and in that way reduces vertebral compression fracture with only modest effects on bone quantity.

ANALGESIC EFFECTS OF NS-SCT

A unique (among currently available osteoporosis therapies) primary pain relieving effect of S-CT following an acute compression fracture in the spine has been described; such a pain relieving effect may be mediated through either a stimulation of beta-endorphins, or through pain receptors in the brain. Evidence for the analgesic effect of S-CT—currently its principal non-bone application—originally emerged during clinical use of the hormone in patients with classic CT indications, such as Paget's disease of bone (45), osteoporotic fracture syndrome (46–48), and tumor metastases of bone (49,50). The search for possible mechanisms of this effect remains inconclusive; on balance it would appear that direct mediation via specific receptors located in the pain perception areas of the brain is the most likely explanation (6).

Whether S-CT may be of value in the relief of chronic back pain related to previous osteoporotic fractures remains undefined; clinically the use of either the injectable (100 IU/d) or nasal spray (200 IU/d) S-CT is warranted following an acute vertebral fracture. Such usage may then allow the individual with the recent compression fracture to be ambulatory due to decreased back pain, and thereby avoid a potential disuse osteopenia that may be associated with the bed rest frequently utilized in the treatment of a recent, painful osteoporotic fracture.

CLINICAL USAGE OF SALMON CALCITONIN

Indications

Indications for the usage of salmon calcitonin include the treatment of postmenopausal women with established osteoporosis (low BMD with or without fracture) in order to prevent future fracture. As well, salmon calcitonin administered, either by nasal spray or parenterally, may be considered for analgesic relief following acute vertebral compression fracture.

Administration

Administration of SCT should be as 200 IU NS-SCT daily, or 100 IU S-CT parenterally, at any time of day, with no relation to meals, and always with at least 1000 mg of calcium daily, and a vitamin D supplement with 400 IU in potentially D depleted individuals (elderly women with limited exposure to vitamin D or sunlight, etc.). There are no current data available on fracture prevention with intermittent usage. The duration of therapy can be through 5 yr; there are no data to confirm fracture reduction beyond that time. It is reason-

able to consider combining NS-SCT with other osteoporosis therapies, particularly bisphosphonates; such a combination has not been studied as to its effect on fracture reduction, but additive or synergistic effects of such combination therapy could be anticipated.

The question of route of administration, coupled with that of optimum dosage forms, is one that has been central to the clinical use of S-CT almost from the outset, and the story of the drug's gradual chemical, pharmacological, biochemical and clinical elucidation and development is paralleled by the story of the continuous search for better, and more patient-friendly, routes of administration. As a peptide, CT was originally destined for administration by the parenteral route only, limiting its usefulness to some extent in view of the need for regular treatment and the increasing reluctance of patients to submit to frequent injections, except in compelling cases. Some alleviation of this problem was achieved with the development of an intramuscular and then subcutaneous injection that caused the patient relatively little discomfort.

To further improve this aspect and with the aim of enabling the patient to self-administer the drug—as well as to reduce the incidence of adverse reactions occurring with the injectable forms of S-CT (see below) and thereby increase the safety of its use—efforts were made over many years to develop alternative dosage forms. The first of these was a nasal spray, which was introduced in 1987 and is now firmly established as the form of choice in most countries of the world.

Pursuing the same objective of improved patient convenience, it was decided some 15 yr ago to develop a technology that would enable S-CT—and other peptides—to be given orally. This exciting project has now resulted in the very promising oral form of S-CT currently under evaluation, probably for use as a complementary form to the nasal spray, as noted below.

Adverse Effects

Adverse effects include only rhinitis for the NS-SCT; for the parenteral formulation facial flushing and/or nausea and vomiting can occur following injection. For this reason the parenteral formulation is rarely used at this time.

Monitoring

Monitoring the therapeutic response to S-CT includes a measurement of BMD at the spine/hip 2 yr after initiation of therapy to assure therapeutic response. A measurement of a bone marker of resorption (a telopeptide most frequently) 3 mo after therapy initiation would also be indicated. As the increase in BMD with SCT is modest, preservation of BMD (no significant loss), in combination with a significant reduction in a marker of bone resorption (15% reduction in the serum telopeptide at 3 mo, or 30% reduction in the urinary telopeptide at 3 mo [51]) would assure adherence to therapy and a potential therapeutic response.

Comparison to Other Therapies

Comparison to other therapies includes the ease and convenience of administration of NS-SCT, and its tolerability and safety. Whether or not there would be increased compliance with NS-SCT due to its tolerability is unproven.

Cost

Cost for 200 IU of NS-SCT daily for 1 yr would be about \$800–900.

THE FUTURE FOR S-CT

Future trials with S-CT need to assess its efficacy in the prevention of hip fracture, in corticosteroid induced osteopenia, in male osteoporosis, and potentially in the prevention of significant bone loss in immediately postmenopausal women. A preventive indication for S-CT due to its safety, tolerability, and acceptability is reasonable, but another formulation of S-CT (other than parenteral or nasal spray) may be necessary for patient acceptability as prophylaxis in women who have not yet developed significant bone loss or fractures. In this regard projects are ongoing with an oral formulation of S-CT, which would provide improved patient acceptability and convenience for prophylaxis.

In terms of the development of an oral form of S-CT, probably for usage as a complementary form to the currently available nasal spray, the basic principle of the technology involves the use of a specific carrier that would transport S-CT across the gastrointestinal tract wall. To date considerable pharmaceutical, pharmacokinetic and biological work has been performed, with relevant results obtained with several oral dosage forms (solution, capsule, and tablet). As an example, following a number of toxicity studies a Proof of Concept study (with ascending single dosages) was performed in 40 healthy volunteers to confirm relevant S-CT absorption to what had been found in animals. C_{\max} levels were also relevant, showed acceptable variability, and were similar to those obtained after subcutaneous injection of 50 IU (C_{\max} approx 100 picogram/cc). In addition, safety and tolerability data did not reveal any impediment to continued development of this oral form of S-CT. To further optimize the dosage form and to assess pharmacodynamics, tolerability, and clinical response a multiple dose safety and tolerability study in healthy volunteers ($n = 42$) is currently ongoing. An oral form of S-CT would be a useful tool for osteoporosis management, potentially both prevention and treatment, promising high compliance and safety. Potentially the technology involved could also prove to be the answer to the problems of oral delivery for other peptides and other poorly absorbed drugs with a wide safety margin.

As well, studies are needed to elucidate, and to confirm, the mechanism of action of S-CT in reducing pain following compression fracture.

S-CT IN THE TREATMENT OF OSTEOPOROSIS

In conclusion, S-CT, currently utilized primarily as NS-SCT, has proven efficacy in the reduction of spine fracture. As well, a role for calcitonin in pain relief following an acute compression fracture is reasonable. Salmon calcitonin is a valuable therapeutic agent for the treatment of postmenopausal osteoporosis.

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The Mechanism of Action of Nitrogen-Containing Bisphosphonates

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INTRODUCTION

Bisphosphonates (BPs) are highly effective inhibitors of bone resorption and have become the most widely used drugs for the prevention and treatment of osteoporosis. BPs are analogs of pyrophosphate (Pi-O-Pi), in which the oxygen has been replaced by a carbon (Pi-C-Pi), and various side chains attached to the central carbon at either of two sites (R1 and R2) give rise to a large family of BPs, many of which inhibit bone resorption. One of the moieties most often seen in clinically used compounds is a hydroxyl group attached at R1, which increases the affinity of the compound to calcium hydroxyapatite, (1,2). At the R2 site there is a side chain that varies from compound to compound and is responsible for the pharmacological and toxicological differences between BPs. The Pi-C-Pi moiety itself has profound effects on absorption and tissue distribution and plays a critical role in the molecular mechanism of action. Binding to hydroxyapatite also confers several important common pharmacokinetic properties to this class of compounds.

BISPHOSPHONATE PHARMACOKINETICS (PK)

The pharmacokinetics of BPs are strongly influenced by the Pi-C-Pi moiety. The charge and bulk of this moiety prevents it from readily crossing the lipid bilayer of cell membranes. This limits absorption in the gut to around 1% and curtails distribution to most organs outside of bone (3). Absorption is further reduced by food, especially in the presence of calcium. This property requires that oral BP administration occur on an empty stomach without subsequent ingestion of any food (for a period of at least 30 min

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for alendronate [ALN]). Twenty-four hours after administration, less than 3% (0.03% of original dose) can be recovered from all organs combined except bone, where it is retained, and kidney, the major site of excretion (4). There are no enzymes in the organism capable of cleaving the P-C-P bonds, thus minimizing the possibility of metabolism (none was observed for ALN). However, other BPs such as clodronate (CL2) were shown to be metabolized into ATP analogs by substituting for the $\beta\gamma$ phosphates (5,6).

After reaching the bloodstream by either oral or parenteral administration, the clearance of BP is relatively rapid ($t_{1/2}$ about 1 h) via uptake in bone and the combination of excretion and secretion via the kidney. The capacity of skeletal hydroxyapatite for BP uptake is enormous, and BP is extracted from the blood that perfuses bone. Blood perfusion of the skeleton and mineral exposure are not uniform and thus skeletal uptake varies from site to site (7). The highest perfusion occurs at sites of active bone turnover (resorption and formation), and that is the basis for the diagnostic use of methylene diphosphate technetium (Technetium-99m) bone scanning (8,9). On average, BP uptake is higher in cancellous bone (where bone turnover is approx 30%/yr) relative to cortical bone (where bone turnover is approx 3%/yr). Uptake is highest at sites of active bone growth or remodeling within each of these compartments. Similarly, uptake is increased at sites of bone destruction (e.g., bone metastases or active lesions of Paget's bone disease).

Elimination of BPs from the organism occurs virtually entirely through the kidney and can be divided kinetically into several phases (7,10). The rapid phase, occurring within approximately the first 36 h, amounts to 40–50% of the absorbed or injected BP. The balance is retained in the skeleton. Release from the skeleton occurs as part of skeletal turnover, yielding a terminal elimination half-life in humans of over 10 yr, with shorter half-lives in various animal species.

Thus, the pharmacokinetics of BPs (as learned primarily from extensive ALN studies) in humans involves: rapid, but limited (<1%), absorption in the upper gastrointestinal tract; rapid ($t_{1/2}$ of <2 h) clearance from the circulation via uptake in the skeleton (about 50%) and excretion in the urine (about 50%); virtual lack of uptake by any other tissue; and a slow terminal elimination time from the skeleton ($t_{1/2}$ >10 yr) (10,11).

MICROSCOPIC LOCALIZATION OF BPS IN BONE

Autoradiographic examination of histological bone sections following administration of a pharmacologically effective dose of ALN to mice or rat pups showed preferential localization on bone resorption surfaces beneath osteoclasts (12–14). Radioactive ALN was also found on resting bone surfaces albeit at lower levels. The density of label on resorption surfaces, relative to formation surfaces, was estimated to be approx 8:1. With increasing doses, a larger proportion of ALN label localized on bone formation surfaces but only when dosing exceeded pharmacologically relevant levels. However, a pharmacologically effective dose of etidronate (EHDP), which is higher than that of ALN owing to its lower potency, showed approximately equal labeling density on resorption and formation surfaces (14). There are two likely explanations for the initial preferential uptake of BP on bone resorption surfaces: exposure of the bone mineral and higher vascular perfusion. Since BPs are not likely to cross an impermeable sealing zone, it was assumed that they localize on the exposed surfaces prior to osteoclast adherence. However, recent observations by Stenbeck and Horton (15) suggest that the sealing zone of osteoclasts may not be impermeable and could allow penetration of

molecules with a molecular weight at or below 10,000 daltons (ALN is 248 daltons). The selective localization on resorption surfaces, characteristic of the potent nitrogen-containing BPs, is reduced or lost when the dose is increased (as in the case of EHDP) and overflow to formation surfaces takes place.

BISPHOSPHONATE EFFECTS AT THE TISSUE LEVEL

The best documented action of BPs on bone is inhibition of bone resorption. This has been demonstrated in many animal models as well as in humans. The most rapid manifestation of BP inhibition of bone resorption at the tissue level was observed in growing rats (*16*) as epiphyseal clubbing. This is due to inhibition of the modeling associated with longitudinal growth, and results in retention of the secondary spongiosa and cortical bone at the metaphysis. The bone loss caused by accelerated cancellous bone resorption in estrogen deficient female rats is also suppressed by BP treatment (*17–24*). Furthermore, the resorption-driven increase in bone turnover caused by estrogen deficiency (quantified histomorphometrically) was shown to be reduced by BP treatment in rats and baboons (*25,26*). As a consequence, there is an increase in cancellous bone volume associated with preservation of trabecular number and a reduced separation between trabeculae (*16,21,24–27*).

Relatively soon after initiation of treatment (*28–33*), the suppression of bone resorption is reflected by a reduction in the level of collagen C-terminal or N-terminal telopeptides. The suppression of resorption is followed by a reduction in markers of bone formation, reflective of the coupling of formation to resorption and an overall reduction in bone turnover. The first consequence of this reduction on turnover is filling of the remodeling space, (i.e., completion of bone formation that was ongoing when inhibition of resorption was initiated). Second, there is a change in the overall balance between resorption and formation. These two processes result in a net accumulation of bone and an increase in bone mineral density. This is most pronounced in the more rapidly remodeling cancellous bone of the vertebrae and trochanter, but it also occurs in the femoral neck. The third consequence of the decreased bone turnover is more complete mineralization and more “perfect” mineral crystals (*26,34–37*). This effect on mineralization is not induced by EHDP. All three effects result in an increase in bone strength that is reflected in clinical trials by a reduction in fracture risk (*38–48*).

Thus, BPs at the tissue level reduce bone resorption and bone turnover, and produce a positive bone balance and better mineralization, eventually resulting in a reduction in fracture risk (*44,49,50*).

CELLULAR EFFECTS OF BISPHOSPHONATES

It is now generally accepted that inhibition of bone resorption results from the effects of BPs on the resorbing osteoclasts, most likely through direct actions on these cells. There is histological evidence for the localization of BPs beneath osteoclasts on surfaces of bone resorption, and there is similar autoradiographic evidence for the presence of ALN inside the osteoclasts 12 h after administration (*14*). The pathway for BP internalization has not been elucidated in detail, but the products of bone resorption are taken up by osteoclasts and are transported through the cell to the opposite surface by the process of transcytosis (*51,52*). This is the most likely avenue for BP penetration into the cell. However, it is still unclear how BPs cross the membranes of the uptake-vesicles to end up in the cytosol, where they seem to be acting.

The fact that BPs act inside the cell is supported by several lines of evidence. Most compelling is that the molecular targets of BPs, both for nitrogen-containing BPs (N-BPs) and other BPs, is cytosolic. In addition, osteoclasts that can not take up material from their surroundings due to a mutation (oc/oc) failed to respond to tiludronate by disruption of the cytoskeleton (actin rings), but disruption occurred when the BP was microinjected into the cell (53). The cellular changes produced by BPs in osteoclasts includes a disappearance of the ruffled border (12). The ruffled border, a hallmark of osteoclast activity, is part of the osteoclast polarization that follows cytoskeleton rearrangement, reflected in the actin-rich ring-like sealing zone. In addition to these morphological changes that are indicative of osteoclast inactivation BPs may also induce osteoclast apoptosis (54–56).

Therefore, at the cellular level BPs are internalized by osteoclasts resorbing a BP-covered bone surface. Inside the cell BPs cause biochemical changes that lead to osteoclast inactivation and eventually osteoclast apoptosis.

MOLECULAR MECHANISMS OF BISPHOSPHONATE ACTION

Over the years, BPs were shown to affect multiple biochemical processes, some of them related to myriad reactions involving phosphate, (e.g., inhibition of phosphatases) (57–61). Although these effects are real and probably occur *in vivo*, they do not adequately explain the mechanism of BP action. They do not predict the potency of BP compounds and do not provide a direct biochemical link to the pharmacological effects of BP. Although there is compelling evidence that the mechanisms highlighted below play a major role in bisphosphonate action, the contribution of other biochemical effects of BPs, such as the inhibition of protein tyrosine phosphatase shown to occur to various degrees by all BPs, or inhibition of H⁺ ATPase shown to occur for tiludronate (62), cannot be excluded.

Compelling evidence implicates two separate molecular targets responsible for the effects of BPs. One is responsible for nitrogen-containing BPs (such as ALN, risedronate [RIS], ibandronate, pamidronate, zoledronate, etc.), and one for non N-BPs (EHDP, CL2, tiludronate), respectively. The non N-BPs, which are considerably less potent than N-BPs, inhibit osteoclast activity by forming toxic ATP analogs produced by substitution of the BP for P-O-P in the tRNA biochemical pathway (5). This has been best documented for CL2 but is very likely for EHDP and probably for tiludronate as well. The fundamentals of ATP analog formation have been reviewed elsewhere (63).

The N-BPs have been the subject of extensive study, in part because they comprise most of the current therapies in use or under clinical development for the treatment of post-menopausal osteoporosis. About 10 yr ago Amin et al. reported that N-BPs can inhibit cholesterol synthesis *in vitro* (64,65). Some compounds were shown to inhibit squalene synthase, a downstream enzyme in the mevalonate to cholesterol pathway. However, others (e.g., ALN and pamidronate) also inhibited cholesterol synthesis without inhibiting squalene synthase, raising the possibility that an enzyme further upstream was also affected. There are currently several lines of evidence, both pharmacological and biochemical, supporting the hypothesis that N-BPs act on the mevalonate pathway enzyme farnesyl diphosphate (FPP) synthase (Fig. 1). As a result, they reduce protein isoprenylation, critically protein geranylgeranylation, ultimately leading to suppression of osteoclastic bone resorption and induction of osteoclast apoptosis.

Studies conducted in several laboratories have identified FPP synthase as the enzyme inhibited by N-BPs. Indeed, human recombinant purified FPP synthase was shown to be

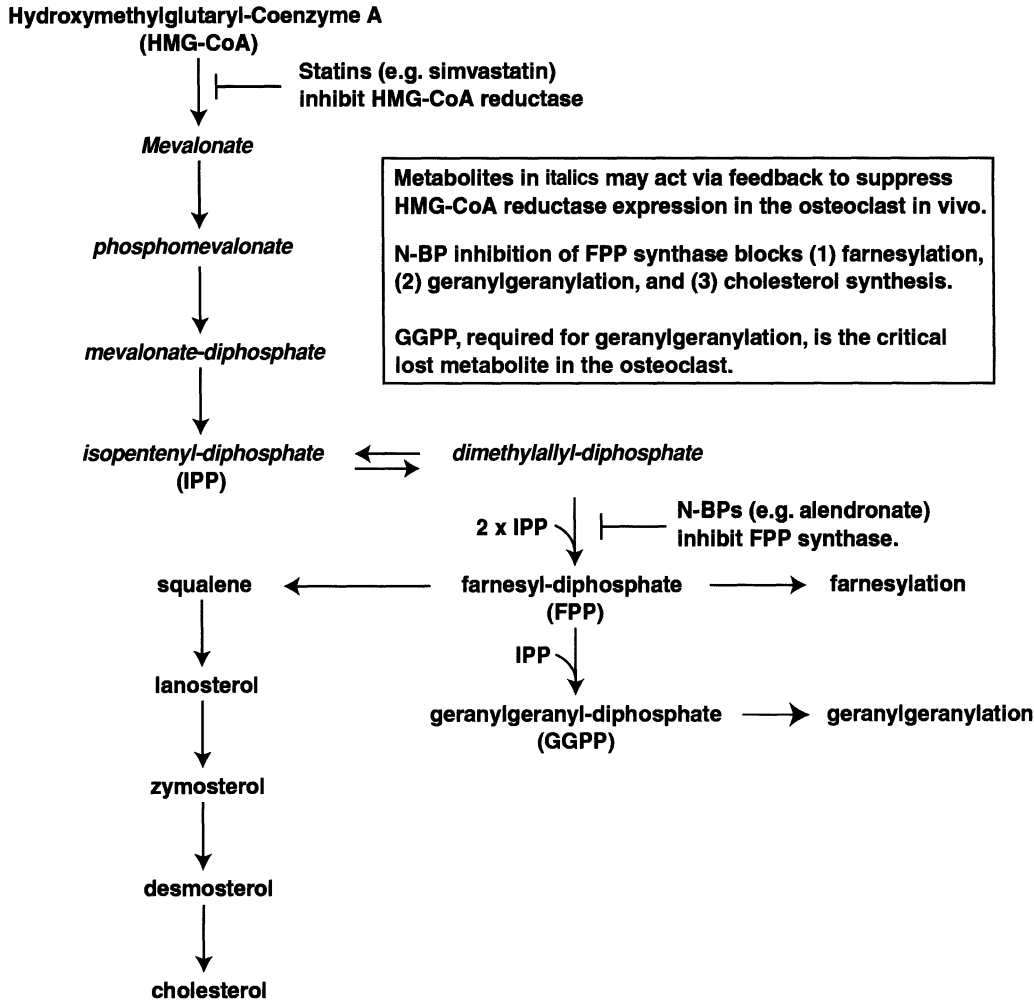


Fig. 1. Schematic of the metabolic pathway targeted by the N-BPs.

inhibited at nanomolar concentrations (IC_{50}) by ALN, pamidronate, and RIS but not by EHDP or CL2 (66). Recent analyses of bacterially-expressed enzyme also showed inhibition (IC_{50}) of FPP synthase by nanomolar concentrations of ibandronate (IBA), incadronate, minodronate and zoledronate (67). These concentrations are below those that have been estimated to be present in the local environment of the osteoclast in vivo, which can reach up to several hundred micromolar (12). A direct correlation between IC_{50} for suppression of FPP synthase and the lowest effective dose for in vivo suppression of bone resorption has been described (67).

The importance of FPP synthesis inhibition as a mechanism for the effects of N-BPs was actually demonstrated prior to the identification of the exact molecular target. That N-BPs reduce isoprenylation in macrophages (68,69) and osteoclasts (66) was deduced from the suppressed incorporation of radioactive label into proteins of molecular weight range around 20–50 kDa, following exposure of the cell cultures to radioactive mevalonate. The explanation for this observation is that the metabolic pathway (mevalonate pathway) leading to formation of isoprenylation precursors (FPP and

geranylgeranyl diphosphate [GGPP]) is essentially unidirectional. Mevalonate is converted to mevalonate diphosphate and then isopentenyl diphosphate (IPP). IPP and an isomer, dimethylallyl diphosphate (DMAPP), act as substrates for FPP synthase, which catalyzes the condensation of two IPPs with one DMAPP to form the fifteen carbon FPP. FPP can lead to cholesterol synthesis, can be the direct substrate for protein farnesylation, or can be further condensed with IPP to form the twenty carbon GGPP (the substrate for protein geranylgeranylation). Therefore the suppression of FPP synthase leads to a reduction in the formation of isoprenylation precursors necessary for both farnesylation and geranylgeranylation.

There is substantial pharmacological evidence that suggests the mevalonate pathway, of which FPP synthase is a component, is critical for osteoclast function and survival. For instance, statins, that inhibit the pathway upstream at the level of HMG-CoA reductase, can mimic N-BP effects (the induction of macrophage or osteoclast apoptosis or the inhibition of osteoclast activity in vitro) (56,68,70). The effects of statins on bone resorption were prevented by the addition of exogenous mevalonate, the metabolite immediately downstream of HMG-CoA reductase. This essentially proved that the action of statins on osteoclastic bone resorption is the result of inhibition of this metabolic pathway. Statin effects are also prevented by the exogenous addition of geranylgeraniol (GGOH), a lipid alcohol precursor of GGPP (70,71). Mevalonate feeds into the metabolic pathway upstream of FPP synthase, the target of N-BPs, and is therefore ineffective at blocking N-BP suppression of bone resorption (70,71). Interestingly, farnesol (FOH), which selectively restores FPP, is also ineffective, probably due to an inability to channel the exogenously added FOH towards synthesis of GGPP rather than cholesterol. Similar to effects of statins, GGOH, but not FOH, effectively reduced N-BP suppression of osteoclastic bone resorption. Thus, even prior to the identification of FPP synthase as the molecular target of the N-BPs critical proof was in place establishing that suppression of the mevalonate metabolic pathway by N-BPs was the mechanism of action of this class of bisphosphonates.

Parallel studies implicate inhibition of FPP synthase by N-BPs in the induction of osteoclast apoptosis (56), as well as in growth suppression of keratinocytes, a cell type used to model the tissue lining of the esophagus (72). Earlier work in macrophages implicated inhibition of both farnesylation and geranylgeranylation in the apoptotic effects of N-BPs (68). However, consistent with the bone resorption response, osteoclast apoptosis induced by N-BPs could be prevented by GGOH but not FOH, implicating geranylgeranylation as the critical process disrupted by the N-BPs (56). More recently, a specific inhibitor of geranylgeranylation, but not farnesylation, has been shown to induce osteoclast apoptosis and suppress bone resorption (73).

In osteoclasts, downstream effector proteins have been identified that mediate effects of BPs on cell survival or growth regulation. One of the biochemical changes identified to be involved in BP induction of apoptosis in osteoclasts is activation of *Mst1* kinase (56). This pro-apoptotic kinase was induced in purified osteoclast cultures by the N-BPs alendronate and RIS, non-N-BPs CL2 and EHDP, lovastatin, treatment with staurosporine or withdrawal of the survival factor M-CSF. This effect was prevented in the case of N-BPs and statins (but not in the case of EHDP, CL2, staurosporine or M-CSF withdrawal) by exogenous addition of GGOH. These observations indicate that the biochemical signaling for BP-induced apoptosis is upstream of *Mst1* kinase activation,

and for N-BPs it is downstream of inhibition of isoprenylation. An intermediary in this process is caspase 3, that can both cleave *Mst1* to form a catalytically active and unregulated kinase, itself activated by the presence of active *Mst1* in the cell (74). Caspase 3-like activity has been observed in the osteoclast (75) and bisphosphonate-induced caspase 3 induction in macrophages was suppressed by GGOH or FOH (76).

Most evidence relating inhibition of FPP synthase to suppression of osteoclast activity has been obtained in vitro. However, recent observations in vivo support the conclusions that the N-BPs affect the mevalonate pathway (77). Following administration of a pharmacological dose of ALN, RIS, or IBA to rats there is significant suppression of the expression of the mevalonate pathway upstream enzyme HMG CoA reductase. The effect is noted in osteoclasts but in no other cells in bone or bone marrow. Under the same conditions, treatment with the non N-BPs CL2 and EHDP has no effect on HMG-CoA reductase expression. Coadministration of simvastatin, the inhibitor of HMG-CoA reductase activity, opposes the ALN induced suppression of HMG-CoA reductase expression in the osteoclast in vivo. This is consistent with the accumulation of a mevalonate pathway metabolite, situated between HMG-CoA reductase (statin target) and FPP synthase (N-BP target), that regulates the expression of HMG-CoA reductase by feedback inhibition.

Taken together, the available data strongly support the hypothesis that N-BP inhibition of farnesyl diphosphate synthase and the consequent reduction in GGPP, required for isoprenylation, is the molecular mechanism for N-BP inhibition of osteoclast activity and induction of osteoclast apoptosis. While certain of the downstream effectors of N-BP action have been identified, the specific prenylated protein(s) responsible for osteoclast inactivation and apoptosis and for keratinocyte growth suppression (discussed below) remain to be identified.

Is there a role for FPP inhibition in the causation of local irritation of the upper gastrointestinal (GI) track, an infrequent clinical occurrence associated with the use of BPs (78–82)? In cultured keratinocytes, (a model of the esophageal lining) (72) there is an absence of the apoptosis observed in previous osteoclast studies. Instead, cell growth suppression is observed at or below concentrations associated with clinical dosing. Given that the stratified squamous epithelium covering the esophagus undergoes rapid turnover as a consequence of keratinocyte growth and differentiation, growth suppression by N-BPs could interfere with a normal process utilized to maintain the integrity of the esophageal lining.

As in studies of the osteoclast, the effects of N-BPs on keratinocytes could be mimicked with an inhibitor of geranylgeranylation and blocked with the addition of GGOH. Unlike in osteoclasts, suppression of cholesterol synthesis is also implicated in growth suppression since the effects of N-BPs can be mimicked with a selective inhibitor of cholesterol biosynthesis and blocked by the addition of LDL-cholesterol. Detailed comparison of two clinically used N-BPs reveal an identical response in each of fifteen molecular analyses of the mevalonate pathway, protein isoprenylation and effects on downstream proteins known to regulate cell growth. This, and the combined geranylgeranylation and cholesterol effects, point to a common mechanism for growth suppression by N-BPs. FPP synthase inhibition is likely the mechanism responsible for irritation of esophageal tissues.

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23

Bisphosphonate Treatment of Osteoporosis

Nelson B. Watts, MD

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INTRODUCTION

Bisphosphonates are avid bone-seeking compounds with potent antiresorptive effects. They share a common chemical structure (Fig. 1) of two phosphonic acids joined to a carbon, with two side chains that can be modified to change the affinity for bone and the antiresorptive potency. They are useful for the prevention and treatment of osteoporosis and other conditions characterized by increased bone remodeling. In addition to their diagnostic use for nuclear bone scintigraphy, bisphosphonates have been applied to the treatment of many conditions of abnormal bone resorption and remodeling, including myositis ossificans progressiva (1,2), fibrous dysplasia (3), heterotopic ossification (4,5), otosclerosis (6), Paget's disease of bone (7,8), hypercalcemia due to spinal cord injury (9), bed rest (10), malignancy (11), hyperparathyroidism (12), and other causes (13–15), bone loss due to thyroid hormone excess (16), corticosteroid excess (17–20), or paraplegia (9), prevention of severe hypocalcemia after surgery for hyperparathyroidism (21), destructive arthropathy (22,23), and skeletal involvement with metastatic cancer (24,25), or multiple myeloma (26). Although most of the clinical applications have been in adults, there is increasing experience on the use of bisphosphonates in children (27–29).

A number of bisphosphonates have come to be accepted for use in the treatment of osteoporosis and for prevention of bone loss in recently menopausal women (Table 1).

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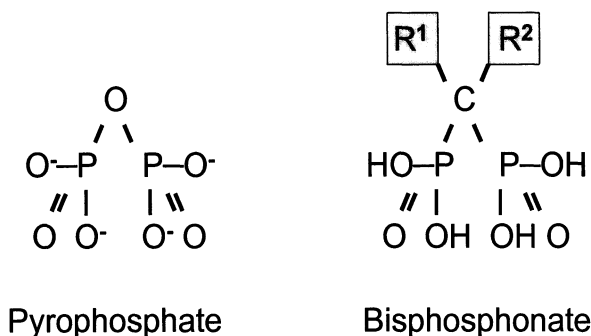


Fig. 1. Structure of pyrophosphate and geminal bisphosphonates. Reproduced with permission from Watts NB. Bisphosphonate therapy for postmenopausal osteoporosis, in: Avioli L ed., *The Osteoporotic Syndrome*, 4th edition, 2000 Academic Press.

In this chapter, the clinical trial data for those compounds that are available in North America will be reviewed.

ETIDRONATE

Clinical Trials

Etidronate was the first bisphosphonate to be investigated for the treatment of postmenopausal osteoporosis. Although its effects on bone seemed generally positive, continuous therapy with etidronate resulted in impaired mineralization of new bone (30). An intermittent cyclical regimen was developed that showed safety and encouraging efficacy in small trials (31–33). The current accepted regimen of intermittent cyclical etidronate for treatment of osteoporosis is 400 mg of oral etidronate (5–10 mg/kg/d) daily for 14 d, repeated every 3 mo. As is true for other bisphosphonates, etidronate must be taken on an empty stomach (with water only). Etidronate can be taken between meals (2 h before and 2 h after eating) or during the night.

Two prospective, randomized, controlled trials showed that intermittent cyclical etidronate therapy significantly increased spinal bone mass and suggested a decrease in the risk of vertebral fractures, especially among high-risk patients (34,35). The observations from these two studies have been extended through 5 yr (36) and seven years (37,38). Postmarketing data with etidronate have shown a reduction in nonvertebral fractures (39) and an excellent safety profile (40).

In addition to the studies for treatment of postmenopausal osteoporosis, intermittent cyclical etidronate has been shown to be effective for prevention of bone loss in recently menopausal women (41,42) and for prevention (17) and treatment (43) of corticosteroid-induced osteoporosis (44). A small, open study has shown increases in bone density in osteoporotic men treated with etidronate (45). Estrogen combined with etidronate produces greater gains in bone mineral density (BMD) than either agent used alone (46).

Side Effects

As mentioned, etidronate given continuously in high doses (10 mg/kg/d for 3 mo or longer) may impair mineralization of newly-formed bone, leading to bone pain and fractures (47–49). No other bisphosphonate has this potential with doses that are used clinically. The intermittent cyclical regimen of etidronate for osteoporosis avoids this problem (50,51). Occasionally, etidronate causes diarrhea, which, when it occurs, it

Table 1
Structures of Bisphosphonates in General Clinical use in North America

	R^1	R^2
Non-nitrogen-containing compounds		
Etidronate	OH	CH ₃
Clodronate	Cl	Cl
Tiludronate	H	SC ₆ H ₃ Cl
Nitrogen-containing compounds		
Pamidronate	OH	CH ₂ CH ₂ NH ₂
Alendronate	OH	CH ₂ CH ₂ CH ₂ NH ₂
Risedronate	OH	CH ₂ -3-pyridinyl
Zoledronate	OH	CH ₂ C ₃ N ₂ H ₃

typically mild. Etidronate does not seem have the potential to cause upper gastrointestinal symptoms that is seen with some nitrogen-containing bisphosphonates.

Current Use

Etidronate is approved in Canada and many other countries for use in osteoporosis, but not in the US. However, it is available in the US for other indications (52). Because of its excellent tolerability and relatively low cost, etidronate is frequently used “off label” in the US for patients who cannot tolerate other oral bisphosphonates. It is remarkably well tolerated.

CLODRONATE

Clinical Trials

Clodronate was the next bisphosphonate in clinical use. Clodronate has been used both orally and intravenously for treatment of postmenopausal osteoporosis (53) and corticosteroid-induced osteoporosis (54). An intermittent intravenous regimen (200 mg infused every 3 wk) (55), an oral cyclical regimen (400 mg daily for 25 d, then 60 d off) (56), and continuous oral therapy (400 mg daily) (57,58) all resulted in spinal bone mineral density significantly higher in treated patients than controls. A higher dose regimen of clodronate, 1600 mg daily, has been shown in one study to reduce new metastases in breast cancer patients (59), however, this has not been confirmed in other trials (60,61). Given orally, clodronate, like other bisphosphonates, must be taken on an empty stomach.

Side Effects

Clodronate is generally well tolerated.

Current Use

Clodronate is not available in the US. In Europe and Canada, where it is available, other bisphosphonates are also available for use in osteoporosis, so clodronate is used mainly for treatment of hypercalcemia of malignancy.

TILUDRONATE

Clinical trials with tiludronate were started shortly after trials with clodronate. Tiludronate is approved in the US for treatment of Paget’s disease (62). Unfortunately,

a large Phase III trial of tiludronate for treatment of osteoporosis was terminated because of apparent lack of effect, possibly because the dose being studied was too low.

PAMIDRONATE

Clinical Trials

Next in development came pamidronate (sometimes called APD, for amino-propylidene diphosphonate), the first nitrogen-containing bisphosphonate to be used. It is approved by the US Food and Drug Administration (FDA) for treatment of hypercalcemia of malignancy and Paget's disease of bone (63), but not for use in osteoporosis. Intermittent intravenous infusions of pamidronate have been used to treat postmenopausal osteoporosis (64), for prevention of bone loss in recently menopausal women (65), and in corticosteroid-induced osteoporosis (66,67). A typical regimen is an initial dose of pamidronate 90 mg, with subsequent doses of 30 mg every third month. The 30 mg dose can be infused over about 60 min (68).

Side Effects

Pamidronate has been associated with severe toxic skin reactions (69,70), and a case of aseptic peritonitis (71). Eye reactions (uveitis, scleritis, episcleritis, and conjunctivitis) have been reported (72–74) with a frequency of about 1:1,000. Oral pamidronate may cause esophageal and upper gastrointestinal problems (75), and intravenous pamidronate causes an acute phase response (fever, myalgias, lymphopenia) in about 1/3 of patients receiving their first dose (see general side effects of bisphosphonates, below).

Current Use

Pamidronate is not approved in the US for use in osteoporosis. It is used "off label" for patients who cannot tolerate or cannot absorb oral bisphosphonates.

ALENDRONATE

Clinical Trials

Alendronate was the first bisphosphonate to be approved by the FDA (1995) for use in osteoporosis. It is approved for treatment of postmenopausal osteoporosis, for osteoporosis in men, for prevention of bone loss in recently menopausal women, and for treatment of corticosteroid-induced osteoporosis in men and women.

Alendronate is a nitrogen-containing bisphosphonate (Table 1) (76). The pivotal study with alendronate involved almost 1000 postmenopausal women who had low bone mass. Alendronate increased bone density in the spine and hip (77). Alendronate 10 mg daily seemed to be the optimal dose as judged by increase in spine bone mineral density. The increases in bone mass after 3 yr of treatment appear to be maintained for at least two years when drug is stopped (78). Of interest, the effect of alendronate (and other bisphosphonates) differs at different skeletal sites, being greatest and occurring earliest at the spine, less at the hip, and minimal at the forearm (Fig. 2).

Although the alendronate Phase III Study was not powered to show an effect on fractures, by pooling all the alendronate patients it was possible to show a 48% decrease in the incidence of new vertebral fractures (6.2% in the placebo group vs 3.2%, $p < 0.05$)

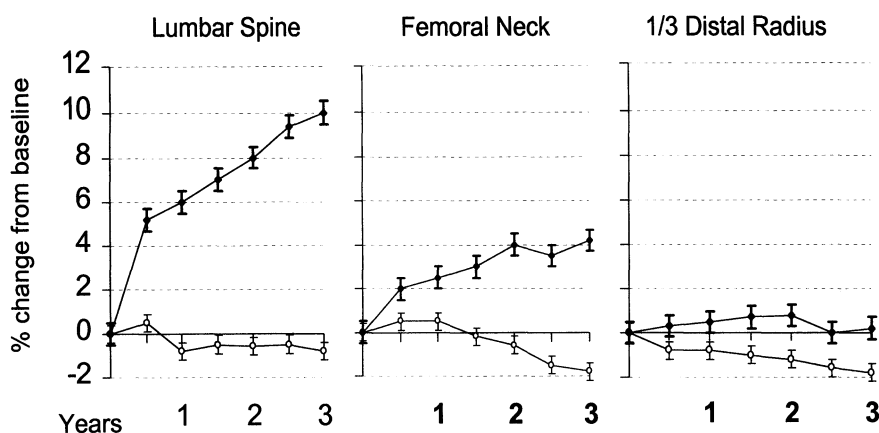


Fig. 2. Mean percent change (\pm SE) in bone mineral density as measured by dual-energy X-ray absorptiometry in the placebo group (open circles) and 10 mg alendronate group (closed diamonds) in women with established postmenopausal osteoporosis. (Adapted from ref. 109.)

(77). To fully assess the effect of alendronate therapy on fractures, the Fracture Intervention Trial (FIT) was undertaken. Postmenopausal women who had low femoral neck BMD (≥ 2.0 SD below the manufacturer's stated mean peak bone mass) were recruited. Women who had prevalent vertebral fractures ($n = 2027$) were assigned to the Vertebral Fracture Arm (79); those who did not have prevalent fractures ($n = 4432$) were assigned to the Clinical Fracture Arm (80). Subjects were randomized to receive either placebo or alendronate (the initial dose of alendronate was 5 mg daily, then increased to 10 mg daily after the second year). The Vertebral Fracture Arm of FIT was planned to last for 3 yr but was terminated a few months early because the reduction in fracture risk was already significant (79). The magnitude of reduction in fracture risk was remarkably consistent: 47% for vertebral fractures, 48% for wrist fractures, and 51% for hip fractures (Fig. 3) (79). The women in the Clinical Fracture Arm of FIT (low femoral neck BMD without vertebral fracture on entry) were followed for over 4 yr. Probably because of an unanticipated problem with the study design (the manufacturer's data base was faulty, resulting in many patients who did not have osteoporosis being included in the trial, thus a lower rate of fractures than anticipated), alendronate did not show an effect on clinical fractures, the primary end-point. However, there was a significant reduction in vertebral fractures in the overall group, and in women whose baseline femoral neck BMD T-score was -2.5 and below by NHANES-III standards, there was a 36% reduction in clinical fractures (80). Similar effects on nonvertebral fractures were shown with alendronate in another trial of over 1900 women with low BMD (81). Therapy with alendronate in FIT was associated with a significant reduction in bed days and days of decreased activity, indicating a favorable effect on quality of life (82).

Alendronate 5 mg daily was shown to prevent bone loss at the spine and hip in recently menopausal women in the Early Postmenopausal Intervention Cohort (EPIC) (83). These observations were recently extended through 4 and 5 yr (84,85),

Estrogen and alendronate 10 mg daily appear to have similar effects on BMD; patients treated with the combination of estrogen plus alendronate show even greater gains in BMD than patients treated with either agent alone (86). Adding alendronate 10 mg daily

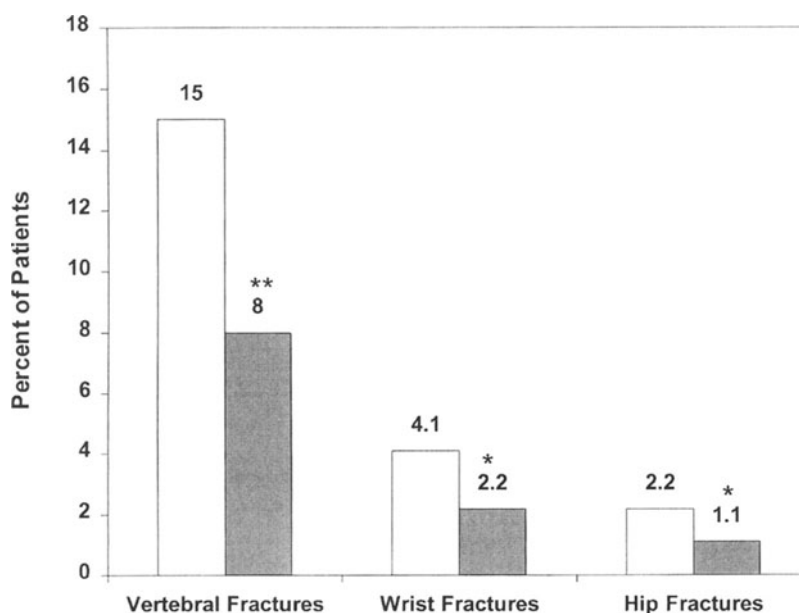


Fig. 3. Percent of placebo patients (open bars) and alendronate-treated patients (shaded bars) in the Vertebral Fracture Arm of the Fracture Intervention Trial (FIT) having new fractures after 3 yr of treatment. ** $P < 0.001$, * $P < 0.05$. (Adapted from ref. 79.)

to women who had been taking estrogen was found to produce significantly greater increases in spine and hip trochanter BMD over 12 mo compared with estrogen alone (87). Alendronate combined with raloxifene produces similar slightly additive effects (88).

In a 2-yr study of men with osteoporosis, alendronate 10 mg daily produced BMD changes similar to those seen in postmenopausal osteoporosis and showed a trend towards reduction of vertebral fractures (89). Alendronate is approved for treatment of osteoporosis in men.

In a study of 477 men and women receiving corticosteroid therapy, alendronate significantly improved spine and hip BMD over 48 wk (18). At the spine, 5 mg alendronate daily had similar effects in men and premenopausal women, but 10 mg daily had a greater effect on spine BMD in postmenopausal women. Alendronate is now FDA-approved for treatment of corticosteroid-induced osteoporosis (10 mg daily for postmenopausal women not taking estrogen, 5 mg daily for men and estrogen-replete women).

Etidronate for osteoporosis was given intermittently because of safety concerns. Although daily dosing of other bisphosphonates, such as alendronate, is safe, it is not convenient or acceptable for all patients. A recent study showed that postmenopausal women given 70 mg alendronate once weekly had an equivalent BMD response at the spine and hip compared with women given alendronate 10 mg daily (90). Tolerability of the 70-mg once weekly dose was as good as or better than 10 mg taken daily.

Side Effects

Although the tolerability of alendronate in clinical trials was good (no different from placebo), when the drug came into general use, serious esophageal events (rare, $\sim 1/10,000$) (91) and more frequent but less serious tolerability problems became apparent (92,93).

To minimize esophageal irritation, alendronate should be taken with 8 oz of water (to be certain that the tablet passes through the esophagus and into the stomach) and the patient should remain upright (seated or standing) until after eating to avoid reflux of drug into the esophagus. Alendronate should not be given to patients who cannot remain upright, to patients who have active upper gastrointestinal symptoms, or to patients who have delayed esophageal emptying (e.g., strictures, achalasia, or severe dysmotility). It should be discontinued if such problems develop during its use (e.g., therapy should be stopped if the patient becomes temporarily bedfast or develops difficulty swallowing, retrosternal pain, or new or increased heartburn).

Current Use

Alendronate is widely employed for its approved indications. The recommended dose for prevention of postmenopausal osteoporosis and for treatment of glucocorticoid-induced osteoporosis in men and estrogen-replete women is 5 mg daily (or 35 mg weekly). For treatment of postmenopausal osteoporosis, for men with osteoporosis (not due to glucocorticoid use), and for estrogen-deficient women with glucocorticoid-induced osteoporosis, the dose is 10 mg daily (or 70 mg weekly).

RISEDRONATE

Clinical Trials

Risedronate is a nitrogen-containing pyridinyl bisphosphonate (94). Its effectiveness was shown in two pivotal studies of over 3600 women with low BMD and prevalent vertebral fractures (95,96). The primary end-point in these two trials was new vertebral fractures, which were reduced by 41–49% (Fig. 4). The effect of risedronate for decreasing the risk of new vertebral fracture was significant after only 1 yr of treatment (97). Nonvertebral fractures, a secondary end-point in these studies, were reduced by 33–39%, the latter being statistically significant ($P=0.02$) (Fig. 4) (95). Risedronate therapy significantly increased BMD at the spine and hip.

In the largest prospective, randomized trial of osteoporosis therapy to date, involving almost 9500 women, treatment with risedronate produced a significant reduction in hip fractures in postmenopausal women who had low bone mass (98). Of interest, a subset of elderly women who were enrolled in the risedronate hip fracture trial because they had clinical risk factors for fractures (but not necessarily low bone mass) did not show a benefit.

Risedronate has been shown to prevent bone loss in a study of 383 recently menopausal women (99).

Risedronate 5 mg daily was shown to be effective for prevention of corticosteroid-induced bone loss in a study of 228 patients beginning corticosteroid therapy (19) and to increase bone density in a study of 290 patients who had been treated with glucocorticoids for an average of 5 yr (20). In a post-hoc analysis of data from these two trials, a significant 70% reduction in new vertebral fractures was shown (100).

Side Effects

In clinical trials of almost 16,000 subjects, adverse events with risedronate were no different from placebo. The tablet travels quickly through the esophagus (101). Risedronate has only recently been introduced to the market (April, 2000). So far it appears to be well tolerated.

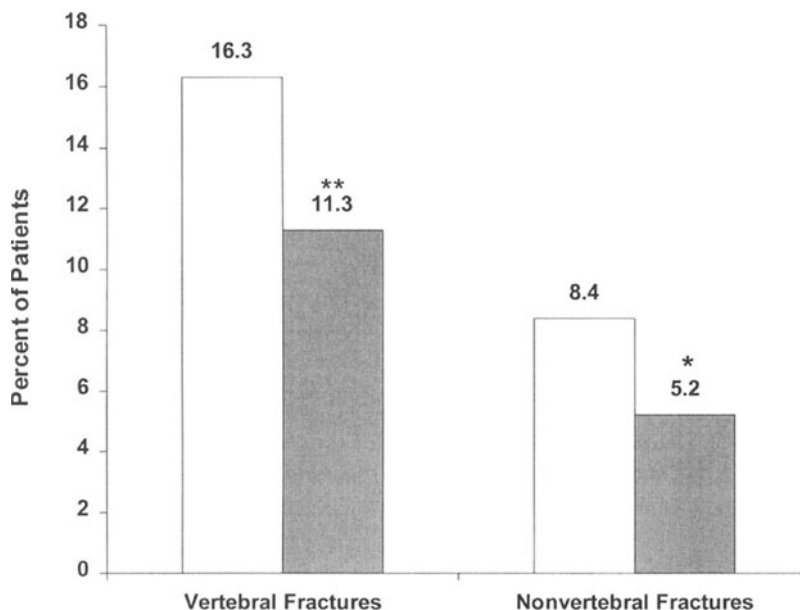


Fig. 4. Percent of placebo patients (open bars) and risedronate-treated patients (shaded bars) in the Vertebral Effectiveness of Risedronate Therapy (VERT) study having new fractures after 3 yr of treatment. ** $P = 0.003$, * $P = 0.023$. (Adapted from ref. 95.)

Current Use

Risedronate is approved for prevention and treatment of postmenopausal osteoporosis and for prevention and treatment of corticosteroid-induced osteoporosis. The dose is 5 mg daily for all of these indications. Although in the US, dosing instructions are the same for alendronate (102) and risedronate (103), risedronate may offer some flexibility in dosing. Pharmacokinetic data show similar systemic levels when risedronate is taken 2 h before or after a meal or at least 30 min before retiring in the evening compared with after an overnight fast (104).

BISPHOSPHONATES IN DEVELOPMENT

A number of other compounds have seen some clinical use, including olpadronate and neridronate. Compounds in the late stages of clinical development include ibandronate (105,106) and zoledronate (107). Ibandronate was studied in an intermittent regimen, up to 1 mg every 3 m by intravenous injection. Despite an increase in BMD, the effect on fractures in this trial was not significant (108). A study using oral ibandronate should be completed soon. Zoledronate is being developed primarily for use in oncology.

COMPARING AGENTS, JUDGING EFFICACY

There are no head-to-head trials comparing one bisphosphonate with another for treatment of osteoporosis or comparing bisphosphonates with other treatments (except using surrogate markers such as changes in BMD or biochemical markers). It is tempting to look at increases in bone density in different trials. However, the changes in bone density using the same agent in different trials can vary by 20–30% (95,109–111).

Nitrogen-containing bisphosphonates may have a greater effect on BMD than non-nitrogen-containing compounds. There has been a small study showing a BMD response to alendronate in patients whose BMD failed to increase with etidronate therapy (112), and another showing an increase in BMD with alendronate treatment following etidronate treatment (113).

There is a weak correlation between the magnitude of increase in BMD after treatment with antiresorptive drugs and the reduction in the rate of vertebral fractures (114). In the randomized trials of bisphosphonates, the confidence intervals surrounding the vertebral fracture risk reduction are too broad to permit conclusions about relative effectiveness. In a retrospective analysis of treated patients from FIT (115), Hochberg et al. concluded that among treated patients, the greater the gain in BMD, the greater the reduction in fracture risk. However, this relationship was not consistent at different time points or at different skeletal sites. Based on available data, it does not appear that change in BMD can be used to judge the relative anti-fracture effectiveness of different bisphosphonates.

For individual patients, however, treatment with bisphosphonates usually results in measurable changes in spine BMD within a year and measurable changes at the total hip within 2–4 yr. Biochemical markers of bone turnover (e.g., serum bone-specific alkaline phosphatase, urine collagen cross links such as N-telopeptides [NTx] and deoxypyridinoline) show measurable decreases within 3–6 mo.

GENERAL SIDE EFFECTS AND TOXICITY

There has also been concern that potent bisphosphonates might turn off remodeling completely, leading to “frozen bone.” There is no evidence that this actually occurs. Fracture healing does not appear to be a problem with low doses of etidronate (116), with alendronate (117), or with any of the newer bisphosphonates.

Acute-phase reactions (fever, myalgias, lymphopenia, etc.) occur in about a third of patients receiving their first dose of intravenous nitrogen-containing bisphosphonates (118,119), but rarely recur with repeated administration (120). Hypocalcemia may also occur with rapid parenteral administration of bisphosphonates; it is infrequent and usually mild (121,122). The degree of hypocalcemia caused by bisphosphonates may be worsened if they are given along with aminoglycoside antibiotics (123).

The main side effects of bisphosphonates occur in the gastrointestinal tract. Oral pamidronate is known to cause upper gastrointestinal side effects (nausea, dyspepsia, inflammation or ulcers of the esophagus, stomach, and duodenum) (63,75,118,120). In the clinical trials with alendronate, another nitrogen-containing bisphosphonate, the incidence of gastrointestinal side effects was low (91), however, problems were noted once the drug was in general use (124), some likely the result of errors in administration or in patient selection. Serious problems (esophageal erosions, ulceration, or bleeding) have been reported in about 1 in 10,000 alendronate users (91). Nonserious but troublesome symptoms may be seen in as many as 10–15% of patients who receive alendronate, with symptoms often beginning after the first dose or two.

REMAINING QUESTIONS, UNRESOLVED ISSUES

Is one bisphosphonate more effective than another? For reasons give above, that would be difficult to answer. It would require a head-to-head study with a fracture end-point. There appear to be more “nonresponders” with etidronate (which does not contain a nitrogen) than with alendronate (a nitrogen-containing bisphosphonate) (112), and those “nonresponders” to etidronate usually experience an increase in BMD with alendronate.

Is one bisphosphonate better tolerated than another? Again, this would require a head-to-head study to answer. The available evidence suggests that few patients have tolerability problems with etidronate. Perhaps 10% of patients treated with daily alendronate using the original formulation have gastrointestinal symptoms; the new formulation or the once weekly dosing regimen may be better tolerated. Although clinical experience with risedronate has been limited, tolerability seems good so far.

When should combination therapy be used? Several classes of agents have proven effectiveness in reducing osteoporotic vertebral fractures. Although all are considered antiresorptive agents, mechanisms of action differ, raising the possibility that combining agents with different mechanisms of action might produce greater benefit than a single agent. Several studies have shown that combining a bisphosphonate with estrogen (46,86,87,125) or raloxifene (88) produces greater gains in bone mass than monotherapy. None of these studies are large enough to determine if there is a greater reduction in fracture risk with combination therapy; given the wide confidence intervals seen in fracture reduction trials, it is unlikely that such data will ever be available. Combining agents obviously increases the cost of treatment and the likelihood of side effects. There is also a possibility that bone turnover could be oversuppressed, resulting in "frozen bone." For these reasons, combination therapy with antiresorptive drugs should be reserved for patients who have severe osteoporosis, and perhaps those who fail to respond to a single drug. Combining an anabolic agent (e.g., parathyroid hormone) with a bisphosphonate presents a model that is theoretically more appealing than the simultaneous use of two antiresorptive medications (126,127).

SUMMARY AND CONCLUSIONS

Bisphosphonates are safe and effective agents for treatment and prevention of osteoporosis. They are the best studied of all agents for osteoporosis in terms of efficacy and safety. They increase bone mass primarily at the lumbar spine but also at the proximal femur. In patients who have established osteoporosis, they reduce the risk of vertebral fractures. Bisphosphonates are the only agents shown in prospective trials to reduce the risk of hip fractures and other non-vertebral fractures. Alendronate and risedronate are approved by the FDA for prevention of bone loss in recently menopausal women, for treatment of postmenopausal osteoporosis, and for prevention (risedronate) and treatment (alendronate and risedronate) glucocorticoid-induced osteoporosis. Other bisphosphonates (etidronate for oral use, pamidronate for intravenous infusion) are also available and can be used off-label for patients who cannot tolerate approved agents. Bisphosphonates combined with estrogen or raloxifene produce greater gains in bone mass compared with single-agent treatment; whether there is a greater benefit of combination therapy on fracture risk is not clear. Combining a bisphosphonate with calcitonin is probably safe, though data on response is lacking.

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CONTENTS

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INTRODUCTION

Small clinical trials using the synthetic fragment human parathyroid hormone (hPTH) 1–34 in the early 1970s suggested PTH could be used as an anabolic therapy for osteoporosis (1,2). Since these initial clinical trials, numerous studies in animal models and humans have demonstrated that PTH anabolic effect is dependent on its intermittent administration. Continuous infusion of PTH results in decreased bone mass due to a change in bone balance to favor bone resorption (1–6). Emphasizing the complexity of the skeletal response to PTH, prolonged continued exposure to PTH as a consequence of hyperparathyroidism shifts bone distribution from cortical bone to trabecular bone, resulting in thinned cortical bone (1–10). PTH uniformly stimulates bone turnover. Whether this results in bone mass gain, loss or equilibrium, is determined by regimen or the presence of disease. Recent work with transgenic mice expressing a constitutively activated PTH1 G-protein coupled receptor (PTH1R) implicated the up-regulation and distribution of this receptor as a key step in determining outcomes at specific bone sites (11). Despite a wealth of in vitro research on the cell and molecular actions of PTH, we still do not understand the mechanisms resulting in these multiple effects in vivo. Moreover, none of the in vitro models fully recapitulates the anabolic effects of PTH observed in vivo, to result in osteoblast induction and a net increase in matrix proteins.

The aims of this chapter are to briefly review studies of PTH actions at the molecular and cellular level, including regulation of osteoblasts and osteoclasts, and then to discuss the recent preclinical studies of PTH treatment and withdrawal in animals with osteonal bone more closely resembling that of humans.

CELL AND MOLECULAR ACTIONS

PTH Regulation of Osteoblasts

Research of the past decade has reproducibly shown the osteoblast and its progenitor to be the primary target of PTH in vivo. The mechanisms of action are still not well

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understood, although we know that PTH regulates gene expression in osteoblasts supporting synthesis of various proteins involved in bone formation and resorption. While *in vitro* studies have shown inconsistent effects of PTH on bone cell proliferation (12,13), studies in both young and old rats have provided no evidence to support PTH as a stimulator of osteoblast proliferation (14–17). In young rats, there are large pools of proliferating cells underlying the growth plates, the metaphyseal cortical endosteal surface and the diaphyseal periosteal surfaces (18,19), which may be regulated by PTH (20–23). In the absence of evidence to support an initial stimulation of proliferation, the presence of large numbers of proliferating cells *in vivo*, and the fact that these cells can be enabled to differentiate into different lineage cells, suggest that PTH may recruit proliferating cells into the osteoblast differentiation pathway to increase the number of osteoblasts (21–23). In older animals with closed growth plates and few proliferating progenitors (24), it has been speculated that PTH and its analogs increase osteoblast number by stimulating differentiation of quiescent bone surface cells (15). This hypothesis is supported by a number of studies, including electron microscopy (25); thymidine autoradiography (15), and histomorphometry in both humans and animals. The early increase in bone forming surfaces (Fig. 1) is more consistent with stimulation of differentiation of osteoprogenitors, than an initial increase in proliferation (1,26,27). Cyclic AMP (cAMP) has been implicated as the key signaling pathway by which the anabolic responses of PTH are effected upon binding to the PTH1R (1,26,27). In fibroblasts, cAMP signaling activates protein kinase A (PKA) controlled cell growth by abrogating signaling required for detachment of cells, and by inhibiting both progression through the cell cycle and apoptosis. If valid for osteoprogenitors, this could provide mechanisms by which PTH promotes differentiation but not proliferation of bone cells. For a more intensive review of signal transduction, the reader is referred to ref. 28.

An additional mechanism to increase the number of bone cells may be by PTH inhibition of existing osteoblasts (29–31). However, as cells in which apoptosis is inhibited are thought to die more slowly by alternate pathways, it is not clear if conditions that inhibit bone cell apoptosis permit osteoblasts and osteocytes to remain fully functional. It is also not clear if the inhibitory effect on osteoblast apoptosis occurring in metaphyseal secondary spongiosa is a direct effect of PTH, or an indirect effect associated with changes in bone turnover and displacement of hematopoietic marrow by bone mass (32) as a consequence of PTH treatment. In view of the recent observation that osteosarcoma was associated with lifetime treatment of young growing rats with PTH (33), studies are needed of the prolonged effects of PTH on bone cell biology *in vivo*, to determine if this was an effect of the hormone, or a consequence of the increase in bone turnover and changes in bone mass and geometry induced by PTH. The relevance of this phenomenon to the osteonal skeleton of larger mammals and humans remains uncertain. Apart from an early study on the cortical surface of very young, growing rabbits (34), there have been no studies of PTH-regulated osteoprogenitor proliferation and apoptosis in animals in which the response to PTH is independent of the growth and development skeletal processes always present in rats and mice.

The time-dependent changes activated following PTH treatment (Fig. 1), and the implications for the mechanisms operating during induction of the response, compared to those operating after several remodeling cycles have not been well appreciated. During the early phase of the anabolic response, within 1–6 h of injection, PTH regulates many osteoblastic genes mainly through the cAMP-PKA signaling pathway, with the

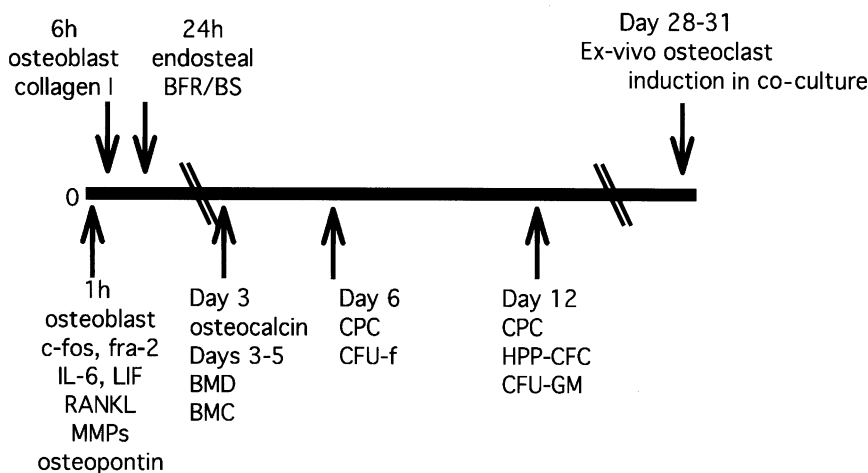


Fig. 1. Selected time-dependent changes in bone and hematopoietic cells after once daily PTH treatment in young rats for up to 31 d. BFR/BS bone formation rate on endosteal trabecular surfaces; MMPs metalloproteases; BMD bone mineral density; BMC bone mineral content; CPC (hematopoietic) committed progenitor cells; CFU-f alkaline phosphatase-positive fibroblastic colony forming units derived from stromal cells, and used as surrogate marker for osteoprogenitor cells; HPP-CFC (hematopoietic) highly proliferative precursors colony forming cells; CFU-GM (hematopoietic) granulocytes and monocyte precursor colony forming units, which are considered to contain the precursors of osteoclasts. Note the immediate activation of osteoblast function, and gene expression of cytokines associated with signal transduction to osteoclasts. When PTH is given as continuous infusion, the time for ex-vivo osteoclast induction is shortened to approx 14 d. Based on data in following work (1–7).

protein kinase C pathway probably also contributing to signal transduction (28,35,36). While it is widely accepted that PTH signals through the PTH1R in bone, newer observations from immunohistochemistry of rat bones have reported the presence of this receptor within the nucleus of bone cells, in close association with the cell cycle sequence in vitro (37,38). The implications of this for understanding PTH mechanisms of action are unknown. PTH-stimulated proteins include transcription factors, matrix proteins required for new bone formation, proteins associated with matrix degradation and turnover, and osteoclast differentiation proteins. Studies in young mice (39) and rats (40) indicate that PTH upregulates cell differentiation in trabecular bone in a dose-dependent manner by transient stimulation of the transcription factors *c-fos*, *c-jun*, and *c-myc*. Of these early response genes, *c-fos* shows the greatest magnitude of change in response to PTH (39) (Fig. 2). In vitro, exposure to PTH (1–34) and PTH-related peptide (PTHrP [1–34]) also rapidly induced *c-fos* gene expression in bone cell lines (41,42).

In young rats, upregulated genes within 1h of PTH injection, also include immediate early genes, IL-6 and LIF and RANKL, RGS2, ADAMS-T, and matrix metalloproteinases, such as collagenase 9 (gelatinase B), while expression of the PTH receptor, histone H4 and OPG are decreased (14,43–48). Interestingly, effects on mRNA expression of osteocalcin, growth factors, such as IGF-I and TGF β and their receptors, are not detectable until a few days after initiation of treatment (23,49,50). Although expression of mRNA for the bone matrix proteins, osteopontin and osteonectin, may be upregulated within an hour of PTH injection in vivo, stimulatory effects on collagen I gene expres-

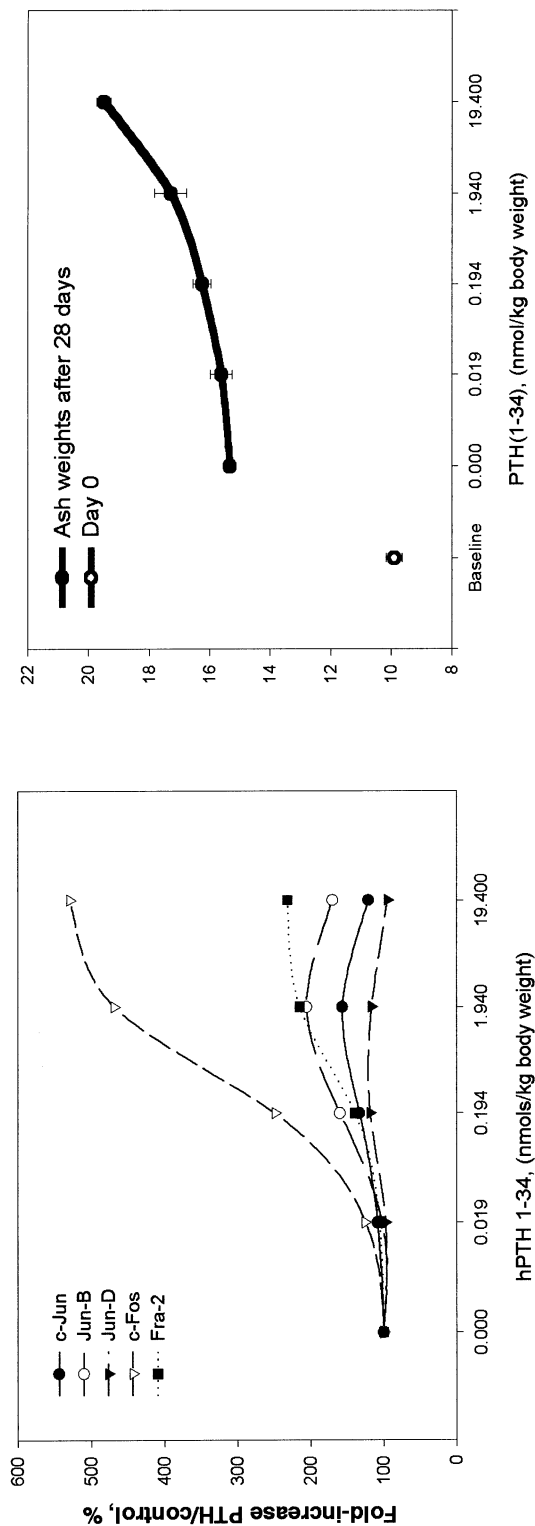


Fig. 2. Left graph: Dose dependent effects of rhPTH 1-34 on expression of AP-I gene family members in femur metaphysis of young male Balb/C mice treated with once daily injections for 3 d. Expression of c-fos, assessed using RNase protection assays, was markedly higher than the other genes; fra-2 expression peaks about 30 min after c-fos has peaked (data not shown). No difference in magnitude was detected when genes were examined after 7 d, compared to 1-3 d (8). Right graph: dose dependent effects of rhPTH 1-34 on bone mass, expressed as ash weight of femurs of young male Balb/C mice treated once daily with rhPTH 1-34 for 28 d. Day 0 value shows ash weight in baseline group, to account for growth of young rapidly growing mice. Data adapted from those reported in ref. 8.

sion require about 6h and translation into increased bone forming surface requires about 24 h, based on histomorphometry and *in situ* histo hybridization (14,23). As microarrays add to our knowledge of changing patterns of gene expression, we will better understand the significance of these changes that represent multiple regulatory pathways.

Much less is known of the PTH-regulated genes associated with continuous infusion or with animal models of hyperparathyroidism. It may be that these gene patterns are those modeled by *in vitro* bone organ cultures and cells. *In vitro*, PTH acts on differentiated osteoblasts to inhibit expression and synthesis of matrix proteins, including collagen 1, osteocalcin and alkaline phosphatase, independently of duration of exposure (51–53). Following continuous infusion of PTH in adult rats, peritrabecular fibrosis and focal resorption were observed, together with hypercalcemia and increased calcitonin (5,6,39,54). It is interesting that over-activation due to a missense mutation in the alpha-subunit of Gs, a protein in a key pathway activated by the PTH1 receptor, was associated with malfunction of mature osteoblasts, manifest as abnormal matrix composition and collagen organization (32,55,56). The fibrosis of hyperparathyroidism and fibrous dysplasia appear to have common features (55). The fibrosis subsequent to PTH regulation of collagen synthesis may be mediated by a changing ratio between IGF-I and IGF-binding proteins, such that IGF-I is suppressed and the IGF-binding proteins dominate in osteoblasts. IGF-I observed in osteoblasts of rats treated with once daily PTH, was not detected in bone lining cells during PTH infusion (57,58). Continuous infusion increased the intensity of staining for IGF-binding proteins, IGF-BP3, BP4, and BP5 (57). These shifts in distribution and magnitude of IGF-1 and IGF-binding proteins associated with different PTH regimens, suggest that these may play a role in cell fate determination of the multipotential progenitors in close proximity to active osteoblasts.

Prolonged exposure to PTH in cultured bone cells alters several nuclear matrix (NM) proteins that mediate nuclear architecture, including NuMA and topoisomerase IIa and IIb, which are structural components of the mitotic chromosome scaffold (59,60). The PTH-induced upregulation of NMP4/NP, another architectural transcription factor which binds directly to the collagen and collagenase promoters, may be a critical mechanism regulating expression of type I collagen (61). These PTH-induced changes in osteoblast microarchitecture via regulation of the NM may modify the profile of transcribed genes that determine a catabolic response (59).

One of the most interesting recent developments in studies of the anabolic effects of PTH has been the consistent finding of upregulated expression of both matrix degrading proteins, such as matrix metalloproteinases (MMPs) and a disintegrin and metalloprotease with thrombospondin motifs (ADAMTS-1) in addition to cytokines associated with regulating matrix degradation and turnover, such as interleukin-6 (IL-6) and IL-11 (14,43,44,47,48). Actions of matrix degrading enzymes to recondition the bone surface may lead to cell detachment resulting in osteoblast apoptosis. Cell detachment *in vivo* is suggested by the finding of a transient increase in apoptosis in proliferating cells and osteocytes of young rat metaphyses during the initial response to PTH (62). Together, these observations of upregulated MMPs and transient increases in apoptosis are consistent with mechanisms activating bone turnover. One consequence of activation of matrix degrading enzymes may be that reconditioned bone surface can serve as an attractant for newly differentiating osteoblasts to increase bone-forming surfaces (anabolic action), or as an attractant for differentiating osteoclasts to continue resorption of old surfaces (catabolic action) (63).

It is interesting that ADAMTS-1 mRNA expression increased by 35-fold within 1 h of a single injection, but by only sevenfold in cells continuously exposed to PTH (47). The different regimens of PTH may govern not only the duration of exposure, but also the intensity of the gene expression response profile. These changes in intensity, together with prolongation in their expression over time, may be sufficient to elicit a different profile of downstream responses.

Regulation of Osteoclasts

While PTH mediates release of calcium from bone surfaces by activation of osteoclasts, recent data suggests that this is an indirect action and that proteins synthesized by osteoblasts responding to PTH, activate osteoclasts. It is currently thought that stromal cells and osteoblast lineage cells regulate osteoclast differentiation through cell-cell contact, by controlling synthesis of OPG and RANKL, the ligand for the osteoclast progenitor receptor, RANK (64,65). These two secreted proteins compete for binding to RANK, a TNF receptor family member (65). If RANKL binding to RANK predominates, as seen following PTH treatment of cultured osteoblast-like osteosarcoma cells transfected with the PTH1 receptor (66), osteoclast progenitors differentiate into osteoclasts (65). In addition, studies in a variety of bone cells lines indicate that PTH downregulates OPG, a potent inhibitor of osteoclast formation and function, via a cAMP/PKA pathway (64). In young rats, mRNA expression for RANKL increases while that for OPG decreases within 1 h of injection (46). The significance of this phenomenon is not known, but may be part of the mechanisms by which PTH activates bone turnover.

The in vitro data (64,65) suggest that increased resorption plays a key role in the mechanisms activated by PTH. It is not known if the activation of osteoclasts following continuous infusion of PTH is a direct effect due to shifts in the ratio of OPG and RANKL expression in osteoblasts and stromal cells. The shift in matrix protein synthesis by osteoblasts to a more fibroblast-like profile (32,54,55) may results in an extracellular matrix feedback signal to activate increased bone turnover. One limitation of these studies is our lack of knowledge of how increased activation frequency may favor formation (anabolic effect of intermittent PTH) or resorption (catabolic effect of continuous PTH). A mathematical model that assumes a longer delay in osteoclast activation (due to a requirement for signals from the osteoblast to osteoclast progenitors) than the delay required for osteoblast differentiation, argues that osteoblast function will predominate with intermittent PTH, while resorption will be greater with continuous PTH (67). This speculation is supported by preliminary unpublished data that show that the ex vivo induction of osteoclasts was delayed in mice given once daily injections of PTH until 28–31 d, whereas under conditions of continuous infusion, increased ex vivo induction of osteoclasts was detected within 14 d (68).

Mediators of PTH Actions

Several candidate agents, implicated as mediators in regulation of the osteoblast axis by PTH, include growth hormone (GH), growth factors, prostaglandins, and 1,25-dihydroxyvitamin D. GH has been evaluated either as a direct regulator of bone cell biology, or as a stimulator of insulin-like growth factor-I (IGF-I), which stimulates osteoblast proliferation and differentiation in vitro (69). Studies in young and old rats suggest that GH, or IGF-I dependent-GH is required for the anabolic effect of PTH

during the “adolescence” phase of skeletal growth but is not necessary after skeletal maturation in this species (70,71). In vitro studies, using bone organ culture or cells isolated from fetal or neonatal animals, suggest that IGF-I may be one of the mediators of PTH effects on skeletal growth and maturation processes via its stimulatory effects on osteoblasts (72). Alternatively, as IGF-I inhibits collagenase (73), IGF-I may mediate a different aspect of the anabolic mechanism, namely regulating the process by which osteoblasts condition the bone surface as a prerequisite to attract osteoclast progenitors to bone. Since skeletal cells secrete the six known IGF binding proteins (IGFBPs) and two of the four known IGFBP-related proteins, there may be additional levels of regulation if IGF-I mediates actions of PTH in vivo (74,75).

In vitro studies have demonstrated PTH-induced regulation of gene expression of other growth factors (e.g., fibroblast growth factor-2 [FGF-2], transforming growth factor- β [TGF- β]) (76,77). However, in vivo studies have not shown these effects until after 4 wk of treatment (50). The effects are dose-dependent and occur at higher doses than those needed to induce an increase in bone mass (50). This delay in upregulation suggests that an increase in growth factors may be more an indication of the highly significant increase in osteoblast numbers and function, rather than primary mediators of the anabolic actions of PTH. In vitro, there is strong evidence to support an intermediary role for prostaglandins in the actions of PTH in bone (53,78). In contrast, treatment of young rats with indomethacin failed to block the anabolic effect of PTH (79), suggesting that prostaglandins are not mediators of the anabolic effects of PTH in vivo. It remains possible, however, that prostaglandins may mediate effects on remodeling and bone turnover in animals with osteonal skeletons.

The vitamin D metabolite, 1,25-dihydroxyvitamin D ($1,25(\text{OH})_2\text{D}_3$), and PTH operate in mutual feedback loops. As PTH raises serum $1,25(\text{OH})_2\text{D}_3$ in vivo, and interacts with $1,25(\text{OH})_2\text{D}_3$ in several molecular pathways in cultured bone cells, $1,25(\text{OH})_2\text{D}_3$ might mediate or contribute to PTH mechanisms of action in vivo. However, experiments in rats have failed to show that $1,25(\text{OH})_2\text{D}_3$ contributes to the anabolic effects of PTH (80–82).

PRECLINICAL STUDIES

Animal Models

Until recently, in vivo data on outcomes after PTH treatment have been predominantly derived from rat studies that have improved our understanding of alterations in modeling drift and bone growth processes. Numerous studies in this model have demonstrated that PTH augments bone mass by stimulating bone formation and increases the resistance of bones to fracture at all sites tested (see reviews by refs. 2 and 83). In mature ovariectomized (OVX) rats, treatment with PTH or one of its analogs increased bone mineral density (BMD) by more than two- to fourfold in the spine and long bones after 6 mo (84,85). In humans, 45–55% of the skeleton is osteonal Haversian cortical bone. In animals with osteonal bone more closely resembling that of humans (e.g., rabbits, dogs, primates), there are effects of PTH that cannot be detected in rats and mice that lack osteonal structure. In osteonal skeletons, PTH-induced bone mass gain, based on dual X-ray absorptiometry (DXA) measures, was in the range of 8–15% in OVX monkeys after 18 mo (86) (Fig. 3) and in osteoporotic women after 2–3 yr (1,33,87,88).

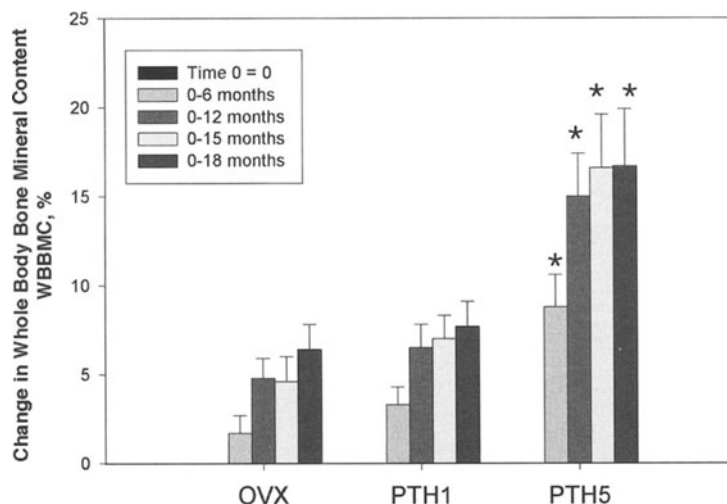


Fig. 3. Change in whole body mineral content of adult, ovariectomized monkeys treated once daily with vehicle (OVX) or rhPTH 1–34 at 1 (PTH1) or 5 (PTH5) mg/kg/d for up to 18 mo. Data are shown as the percent change from baseline for each time point measured, and were regraphed from data in refs. 9 and 10. Values for PTH5 are significantly different ($*p < 0.05$) from those of OVX or PTH1, which were not significantly different from each other at any time point. Note the time course and rapid increase in whole body bone mineral content in PTH5 monkeys.

Anabolic vs Catabolic Effects of PTH

Dosing regimens, duration of treatment and the dose magnitude of *in vivo* studies determine if the outcome is anabolic, in which there is a gain in bone mass due to pronounced stimulation of bone formation, or catabolic, in which stimulation of resorption, combined with a reduction in bone surfaces available for stimulation of formation, result in a net loss of bone mass over time (1,2,89). Between these two extremes, PTH may stimulate activation frequency to accelerate bone turnover, but because there is coordinated upregulation of both formation and resorption, bone mass itself remains unchanged. Even though bone mass is unchanged, bone is redistributed and matrix renewed, as bone architecture may show dramatic changes under conditions of increased turnover. The nature of the response is dependent on whether it is modeling or remodeling that is being studied (32,90–94).

The anabolic effect of PTH has been demonstrated in a wide variety of animals (4,86,95–98) as well as humans (1,33) when administered intermittently. In contrast to rats, in which extensive anabolic PTH dose responses may be conducted without stimulating prolonged hypercalcemia, humans and animals with osteonal skeletons exhibit a narrow dose response within the normocalcemic range (1,2,33,90–93). Mouse strains vary in their responsiveness to anabolic actions of PTH. Although some strains exhibit localized changes in bone mass in response to PTH (39), none exhibit an overall increment in skeletal bone mass (99), suggesting that there is a redistribution of bone within the skeleton necessitating activation of both formation and resorption (100). Catabolic actions of PTH in bone may be induced by continuous infusion, irrespective of animal model (1,3,4,54,90–93,101,102). Given these variations in bone responses to PTH, it

will be necessary to determine the extent to which the molecular and cell mechanisms in rats predict those of humans and animals with osteonal bone structure.

Comparative Effects of PTH on Modeling and Remodeling

In rodents, PTH stimulates bone formation at sites of new metaphyseal bone to modify trabecular three-dimensional geometry. These effects reflect normal growth processes as well as stimulation of bone modeling in which bone formation occurs on quiescent surfaces without prior resorption (89). In humans and animals with osteonal bone structure, remodeling dominates over modeling, so that PTH stimulates significant restructuring of bone via intratrabecular tunneling and intracortical remodeling (effects not observed in rodents), in addition to stimulating apposition of new matrix on endosteal surfaces (89). The overall effect is a positive bone balance in the skeleton (86,95–98,103). These changes observed in osteonal models mimic the processes that have been associated with development and maturation of the skeleton in young humans. Bone turnover due to remodeling, which is an integrated sequence of bone formation and resorption, may blunt the increment of bone gain possible with modeling (90–93). This may explain why the gains in bone mass in human and animals with osteonal skeletons are never as dramatic as the extraordinary increments reported in rats.

Comparative Effects of PTH on Trabecular and Cortical Bone

While the significant anabolic effects of PTH on trabecular bone have been amply demonstrated in many animal models and in humans, the effects of PTH therapy on cortical bone have been controversial. The findings of decreased cortical bone mass in early clinical trials (104–106) and increased cortical porosity in canine models with Haversian remodeling (101,107) have raised concern that PTH treatment might lead to reduced cortical bone mass and strength. Rat studies have uniformly shown that PTH, PTHrP, and their analogs increase cortical thickness and area via endocortical appositional bone growth, to increase resistance of bones to fracture (85,108–111). Recent studies in animals with Haversian remodeling have provided valuable data on the response to PTH at both cortical and trabecular sites which have important clinical implications. Treatment of adult OVX monkeys with hPTH 1–34 at 5 mg/kg once daily for 12 or 18 mo increased whole body bone mineral content (WBBMC) compared to controls within the first 6 mo of treatment (86) (Fig. 3). Observed increases in spinal bone mass and femur neck bone volume were associated with significantly improved biomechanical properties at both sites (112,113) (Fig. 4). Histomorphometry confirmed that the gain in bone mass was due to increased bone surface apposition which occurred early in treatment (97,98,114,115). PTH-treated monkeys exhibited significant remodeling of their trabeculae, such that trabecular number and connectivity increased as the bone formation rate increased (98).

Studies of intact rabbits and the OVX monkey model show complex responses in cortical osteonal bone following PTH treatment (95–97). There is significant stimulation of endocortical bone formation while periosteal formation remains equivalent or slightly higher than controls. Unique to osteonal bone, there is an increase in forming osteons and an increase in porosity of intracortical bone as a reflection of the increase in remodeling transients (95–97). Analyses of the localization of the porosities showed that they occurred predominantly in the endocortical zone, which also exhibits increased apposi-

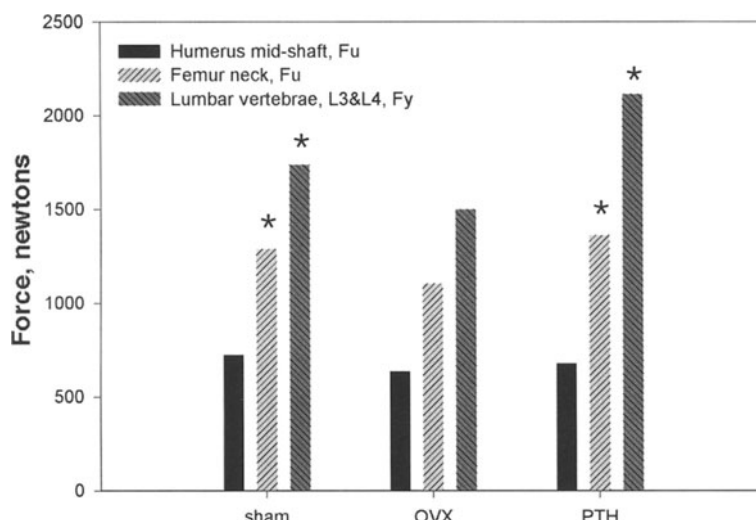


Fig. 4. Biomechanical properties of strength, shown as ultimate force (Fu) for midshaft humerus and femur neck, and as yield force for lumbar vertebrae, L3&L4 resected from adult, sham-operated (sham) and ovariectomized monkeys, treated once daily with vehicle (OVX) or rhPTH 1–34 at 5 mg/kg/d (PTH) for up to 18 mo. PTH values are significantly different ($*p < 0.05$) from either OVX or sham in the femur neck and spine. Data adapted from refs. 11 and 12.

tional bone growth. As this zone is closest to the neutral axis, the cross-sectional moment of inertia, stiffness and ultimate force characteristics of strength remained stable in the mid-cortex of the long bones (95–97).

A clinical concern remains that, if the increase in porosity occurs prior to deposition of PTH-induced new bone formation, an osteoporotic patient with thinned cortical bone may be susceptible to fracture early in treatment. In rats, increases in bone matrix proteins and bone forming surfaces have been demonstrated within 24 h of the first injection of hPTH 1–34 while resorption measures remained unchanged (23) (Fig. 1). More recently, the time sequence of events has been studied in cortical bone of intact rabbits treated with hPTH 1–34 for one remodeling cycle (116). The percent fluorochrome-labeled (new) osteons and endocortical bone formation increased within the first cycle. The increase in porosity was not significant until the end of the first cycle, at 70 d, while the increase in cortical area due to appositional bone formation occurred prior to 35 d. Collectively, these data suggest that PTH “braces” the bone by immediately stimulating formation at both modeling and remodeling sites, so that an initial period of increased susceptibility to fracture would not be predicted.

Consequences of Stopping PTH Treatment

One important issue involving PTH therapy is the possible loss of previously gained BMD after cessation of treatment. Data on the skeletal response to withdrawal of PTH are based primarily on preclinical studies in intact and OVX rats (117–121) and OVX monkeys (97,98,122). In rats, the speed of the withdrawal response may be dependent on either age or the duration of treatment. In young rats, cessation of PTH treatment is followed by rapid loss of the newly added bone due to cessation of the increased bone formation (117,123). In aged rats, there is some evidence for a lasting beneficial effect

Table 1
The Effects of PTH at Each Organizational Level of Osteonal Bone

<i>Cells</i>	<i>Tissue</i>	<i>Organ</i>	<i>Skeleton</i>
1000s up- and down-regulated genes	↑ Bone formation rate	↑ BMC, ↑ BMD	↑ Whole body BMC
↑↑ Osteoblasts differentiate to increase number	↑ Trabecular and endo-cortical bone	↑ Cross-sectional moment of inertia	± Calcium absorption
Regulation of bone cell apoptosis	↑ New osteons		
Delay in ↑ osteoclast activation	↑ Cortical and trabecular remodeling		
<i>Outcomes</i>	<i>Outcomes</i>	<i>Outcomes</i>	<i>Outcomes</i>
↑ Bone forming surfaces Quiescent surfaces decrease	Bone structuring and replacement improves geometry and connectivity ↑ Cortical width	↑ Resistance to fracture ↑ Bone quality	↑ Bone balance and strength

on bone strength after PTH withdrawal (124,125). In OVX monkeys treated for 12 mo, and then examined 6 mo later, the increase in bone mass was smaller, while strength at the spine and femur neck was not significantly different from that of monkeys treated at equivalent doses for 18 mo (112,113,124,125). An unexpected finding was the favorable shift in mineral distribution and size in quantitative computed tomography (QCT) images that correlated with the retention of improved biomechanical properties of bone after withdrawal of treatment (112,113,124,125). It will be interesting to understand if similar phenomena occur in humans following withdrawal of PTH treatment.

Comparisons of the response to withdrawal in ovariectomized rats and monkeys suggest that the incremental gain due to bone modeling during PTH treatment decreases after two or more remodeling cycles (112,113,118–121,125). However, in osteonal skeletons, such as those of monkeys, there is a residual benefit associated with the remodeling processes. Via remodeling in both trabecular and cortical bone (97,98), PTH alters bone geometry, and “rejuvenates” the matrix by turnover and mineralization of new matrix. Following withdrawal, active bone forming surfaces revert to quiescent status (97,98), so that it is likely that, as the bone turnover rate returns to its pretreatment levels, the next fraction of bone to be targeted for turnover will be the older fraction of bone (90–94). One can speculate that there are beneficial effects of PTH on bone quality that will persist for some time after withdrawal of treatment. If the favorable PTH-induced changes in bone quality persist after withdrawal in osteonal skeletons, BMD measures alone may give a misleading evaluation of resistance to fracture. To test this speculation, clinical studies of humans with osteoporosis, previously treated with PTH, are needed to monitor the long-term effects on fracture rate after cessation of PTH treatment.

CONCLUSIONS AND FUTURE DIRECTIONS

The extensive research using *in vitro* approaches and animal models, briefly reviewed here, have provided new insights into the effects of PTH at each organizational level of osteonal bone (Table 1). In light of the clinical trials demonstrating that PTH strengthens bones and increases bone mass at trabecular bone-rich sites with little or no effect on bone mass of cortical bone-rich sites (1,33), the prospects for using PTH and PTH analogs to treat osteopenic states are promising. Preliminary data from a large Phase 3 clinical trial reported a 65–69% reduction in vertebral fractures and a 54% reduction in nonvertebral fractures (33). Nonetheless, there are still unanswered questions that will drive future research. While our knowledge of genetic regulation and signal transduction in bone cells has expanded, there is still limited understanding of the gene patterns and signal transduction pathways that differentially induce and regulate modeling, growth processes, and remodeling *in vivo* to change bone shape or regulate the spatial distribution of bone within a bone organ. Additionally, the cellular and mineralization events associated with PTH withdrawal have yet to be determined. Information is also lacking on the differences in cell and molecular responses to intermittent PTH required to promote osteoblast differentiation and function and those associated with continuous infusion of PTH that activate osteoclast differentiation and function. Lastly, more data are needed in osteonal animal models with thinned cortical bone prior to PTH treatment to fully assuage clinical concerns regarding susceptibility to fracture during the early phases of PTH therapy. New animal models, microarray technology, proteomics, the advances in bioinformatics and in functional pathway mapping should help elucidate much of the mystery still surrounding this bone and calcium-regulating hormone. Note: New data show continuous infusion of PTH upregulated expression of PDGF-A in bone of rats, to stimulate stromal cell proliferation and marrow fibrosis. (Turner R, Lotinun S, Sibonga JD. How hyperparathyroidism causes metabolic bone disease. *Endocrine* 2002;in press.)

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Parathyroid Hormone as a Therapy for Osteoporosis

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INTRODUCTION

Parathyroid hormone (PTH) classically causes bone resorption and bone loss. However, since 1929 (1), it has been known that PTH can also have an anabolic effect on the skeleton. This salutary effect of PTH is the basis for the idea that PTH has the potential to become a therapy for osteoporosis. This chapter will review the effects of endogenous PTH, including lessons learned from primary hyperparathyroidism and the rationale for PTH therapy in osteoporosis. Clinical trials of PTH in osteoporosis, both alone and in combination with other drugs, will also be reviewed.

ENDOGENOUS PARATHYROID HORMONE

In states of parathyroid hormone excess, such as primary hyperparathyroidism, bone loss can occur. Several generations ago in the United States, this catabolic effect on the skeleton gave rise to its description as a disease of “bones, stones and groans.” Since the advent of the multichannel autoanalyzer in the early 1970s, however, primary hyperparathyroidism has presented typically in a milder form, often without clinical manifestations of overt bone disease (2). More detailed evaluation of the skeleton by Silverberg et al. using three-site dual energy X-ray absorptiometry (DXA) has revealed a pattern of bone involvement in these asymptomatic patients. While cortical bone loss is detected routinely, cancellous bone density of the lumbar spine is typically maintained.

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With aging, physiological levels of PTH usually increase (3). This age-related increase in PTH has been viewed by Silverberg and Bilezikian to be potentially beneficial (4) while these same observations have been viewed by Riggs and his colleagues to be potentially detrimental (5). Certainly, in the absence of estrogen, the postmenopausal woman is more likely to experience the effects of PTH whether these changes are adaptive or maladaptive (6). In the distinctly abnormal setting, namely primary hyperparathyroidism, estrogen deficiency in the postmenopausal woman can unmask the disease, presumably because of enhanced sensitivity to PTH (7). Restoring estrogen to the postmenopausal woman may reset the threshold for PTH secretion (8) thereby reducing its skeletal effects (9). This reasoning would lead to the expectation that skeletal responsiveness to PTH should be enhanced in estrogen deficient women with osteoporosis, a point not supported by studies of Tsai et al. (10) or Ebeling et al. (11). On the contrary, PTH dynamics and responsiveness could be reduced in osteoporosis, as shown by reduced nocturnal secretory peaks and greater urinary calcium excretion in postmenopausal women with osteoporosis (12). Moreover, Silverberg and her colleagues have shown that postmenopausal women with osteoporosis do not demonstrate an age-appropriate increase in PTH to a hypocalcemic challenge (13). Finally, Cosman et al. have shown that osteoporotic women have a higher calcium set point than normal pre- and postmenopausal women when these groups are administered hPTH (1–34) (14). These latter observations argue for a putative beneficial adaptive increase in PTH with normal aging, and its loss in postmenopausal osteoporosis.

PRIMARY HYPERPARATHYROIDISM AND CANCELLOUS BONE

In primary hyperparathyroidism there is a loss of cortical bone, but there is a relative maintenance of cancellous bone (Fig. 1) (15). In the subset of postmenopausal women with primary hyperparathyroidism, the preservation of cancellous bone mass in the lumbar spine is even more impressive since estrogen deficiency in the non-hyperparathyroid postmenopausal woman should lead to relative loss of cancellous bone mass. Postmenopausal women with mild primary hyperparathyroidism therefore are generally protected from lumbar spine bone loss due to estrogen deficiency, although in a small proportion of patients, lumbar spine bone density is also reduced.

The protective effect of PTH appears to result from the anabolic effect of PTH on cancellous bone (16–18). Histomorphometric studies have shown, moreover, that in primary hyperparathyroidism there is an absence of the normal age-related loss of bone structure (19). With aging, trabecular number falls and trabecular separation increases, but this relationship is lost in primary hyperparathyroidism (16). Parisien et al. showed not only preservation, but an actual increase in cancellous bone volume in patients with mild primary hyperparathyroidism. In the iliac crest biopsies of 27 patients with mild primary hyperparathyroidism, there was a thinning of cortical bone and an increase in cancellous bone volume; the gain in cancellous bone compensated for loss in cortical bone, resulting in a total bone density that was not different from controls (Fig. 2) (16). When women with primary hyperparathyroidism were compared to normal or osteoporotic women matched for age, bone formation rate was higher, yet cancellous bone volume was the same or higher in women with primary hyperparathyroidism, showing that the anabolic effects of PTH predominate over catabolic effects in the cancellous skeleton (18).

A two-dimensional trabecular strut analysis was performed on 37 iliac crest bone biopsies of patients with primary hyperparathyroidism and compared with control biopsies. When the indices of trabecular connectivity were compared (through comparison

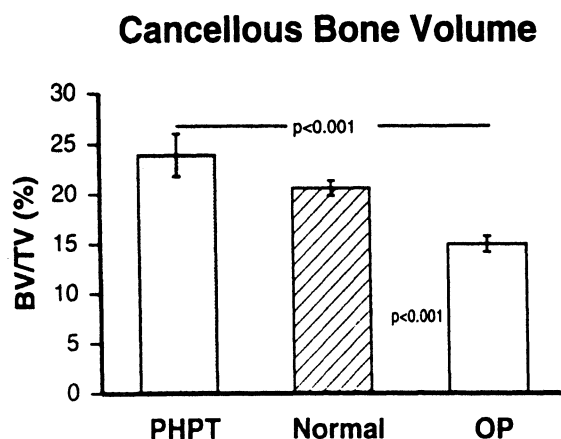


Fig. 1. Cancellous bone volume in groups of hyperparathyroid, osteoporotic and normal women. Values are expressed as mean \pm SEM. Taken with permission from ref. 18.

of the number of junctions between trabecular plates, free ends of termini and the relative length of trabecular struts), it was found that in primary hyperparathyroidism there is not only greater cancellous bone volume and trabecular number, but trabecular plates are better connected (Fig. 3). Thus age-related bone loss in primary hyperparathyroidism is not accompanied by a significant loss of connectivity (17). Another study compared static and dynamic histomorphometric variables of bone formation in 19 hyperparathyroid and 34 healthy postmenopausal women. Hyperparathyroid women had higher indices of bone formation (increases in osteoid width, osteoid maturation time and adjusted apposition rate) and a greater wall width of cancellous bone packets (20).

Fracture Reduction

It is unclear whether the anabolic effect of PTH on cancellous bone in mild primary hyperparathyroidism is associated with a prevention of vertebral fractures. Studies have yielded conflicting results. Vertebral fracture data was compared among 174 patients with asymptomatic primary hyperparathyroidism and a historical control group, and was found to be no higher than expected for the general population (21). Another study examined 90 patients with primary hyperparathyroidism over 11 yr and found that the overall fracture risk was increased prior to the diagnosis of primary hyperparathyroidism, but not thereafter (22). Most recently, 407 patients with mild primary hyperparathyroidism seen over a 28-yr period were found to have an increase in vertebral, distal forearm, rib and pelvic fractures (23). The increase in fracture incidence of the appendicular skeleton would be consistent with the reduced bone density of cortical bone. The apparent increase in fracture incidence in the lumbar spine, an unexpected observation, could be due to ascertainment bias with regard to more careful and diligent monitoring of vertebral status by X-rays in these patients.

RATIONALE FOR ANABOLIC AGENTS IN OSTEOPOROSIS

Anabolic Therapy

All currently approved osteoporosis therapies (through March 2002) are antiresorptive in mechanism, acting primarily by preventing bone loss. Estrogen, raloxifene,

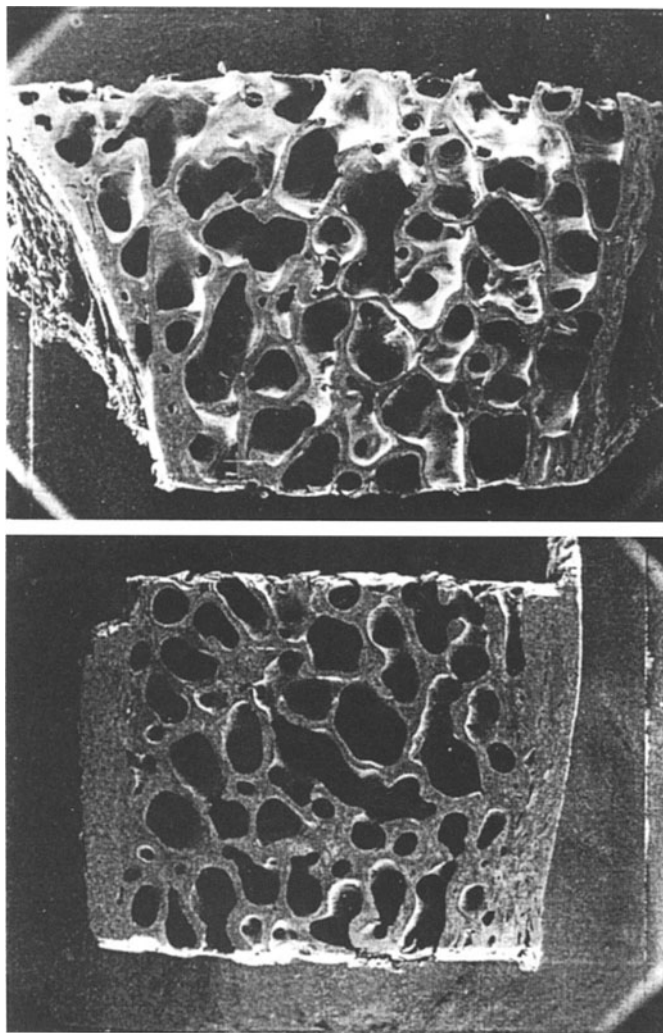


Fig. 2. Scanning electron micrographs of iliac crest biopsies of a patient with primary hyperparathyroidism (top) and a control subject (bottom). Note the thinning of cortices in the patient with primary hyperparathyroidism as well as the maintenance of cancellous bone and trabecular connectivity. Taken with permission from ref. 16.

alendronate, risedronate and calcitonin all inhibit osteoclastic bone resorption and reduce bone turnover (24–26), as demonstrated by their lowering of bone markers of formation and resorption (27,28). These antiresorptive drugs may be associated with a small increase in bone density by reducing the remodeling space, but the increase is typically less than 10% over 3 yr (29,30). They are variably effective in reducing fracture risk. The concept of an anabolic agent is based upon an entirely different physiological process, namely bone formation. Inherent in this concept is the potential for anabolic agents to increase bone mass to a far greater extent than antiresorptives. The potential of these agents to improve bone density more substantially than antiresorptives suggests, in addition, that the efficacious anabolics could reduce fracture risk to a greater extent than efficacious anti-resorptives.

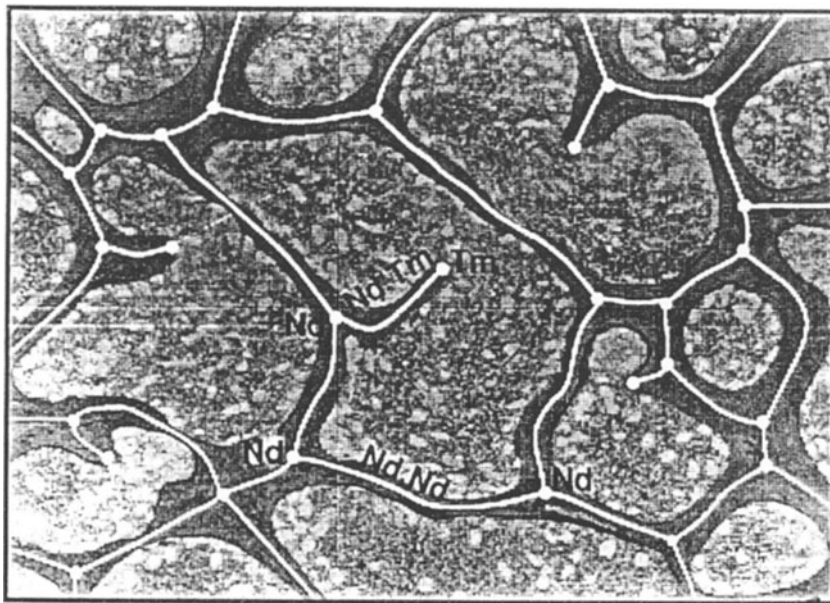


Fig. 3. Photomicrograph of the iliac crest biopsy from a patient with primary hyperparathyroidism using strut analysis. Nodes, free ends and trabecular struts are superimposed on the image. Note the high proportion of nodes compared to free ends. Abbreviations: Nd (node), Tm (terminus), Nd.Nd (node to node strut), and Nd.Tm (node to terminus strut) indicate some of these structures. Taken with permission from ref. 17.

Sodium fluoride was the first of the true anabolic agents to be used in the treatment of postmenopausal osteoporosis. Radiographic increases in bone mass were impressive in early studies with fluoride when administered to osteopenic individuals. Fluoride, in fact, has been used throughout the world for the treatment of osteoporosis, for nearly four decades, although, in the United States, it is not an FDA-approved drug for osteoporosis. One of the reasons for this is the conflicting results of the early randomized prospective controlled trials (RPCTs) in which the use of fluoride was associated with marked increases in vertebral BMD but no change in vertebral fracture incidence. Moreover, the risk of nonvertebral fractures may have been somewhat higher in the trials using a relatively high daily dosage of sodium fluoride, 75 mg (31,32). Side effects consisting of upper gastrointestinal symptoms and a lower extremity pain syndrome were common. The investigation of Meunier et al. was also disappointing when fluoride was used in a somewhat lower dosage (33). Subsequently, Pak and his associates reported on a lower dose, slow release formulation of sodium fluoride in which the pharmacokinetics and serum levels improved the therapeutic/toxicity index. Using this preparation of fluoride, Pak et al. showed a 50% reduction in vertebral fracture incidence along with impressive increases in bone mass (34–36). More recently, Ringe and colleagues have reported positive results with low dose but yet another formulation of fluoride, monofluorophosphate (37–39). Despite the potential for fluoride, especially when used in lower dosages and with more favorable formulations, so as to reduce or eliminate gastrointestinal side effects, consensus about its clinical utility has still not been reached.

Other studies of anabolic agents have also produced mixed results. Androgens, growth hormone, and IGF-1 all have shown promise in selective situations (40) but results in

general have been inconclusive or associated with the potential for substantial long-term adverse consequences. With the exception of androgen replacement therapy for hypogonadal men with osteoporosis, these agents are considered at this time to be highly experimental.

PTH as an Anabolic Agent

The rationale for considering PTH as an anabolic agent for osteoporosis comes from the clinical observations in primary hyperparathyroidism (reviewed above) and a voluminous animal literature reviewed in the companion Chapter 24. Most human (41–43) and animal (44) studies favor the use of low dose intermittent PTH infusion, as compared with protocols associated with chronically elevated levels, to maximize anabolic and minimize catabolic potential.

It is unclear why continuous PTH secretion elicits a different response from intermittent PTH administration. Different theories have been proposed. PTH binds to more than one receptor (45–47), so it is possible that different receptors mediate the anabolic and catabolic responses (48). Another hypothesis is that two distinct second messenger systems each directing different responses are activated by PTH, depending on the pattern of PTH delivery (49).

The anabolic effects of PTH are not limited to the setting of intermittent pulsing: withdrawal of PTH may also increase cancellous bone mass. Parathyroidectomy for primary hyperparathyroidism, for example, will lead to increases in lumbar spine and femoral neck bone density that can exceed 10% (50,51). This bone-building effect of PTH is evidenced by the rapid fall in bone resorption markers after parathyroidectomy, followed by the more gradual decline in formation markers (52), thus shifting bone remodeling toward an anabolic build-up. The increased bone density in the setting of parathyroidectomy may occur because surgery restores the normal pulsatility to PTH secretion (50), and changes levels from being continuously elevated to the more physiologic biphasic circadian rhythm. Another explanation may be the postoperative remineralization that occurs in the enlarged bone remodeling space created by excess PTH (53).

Mechanism of Anabolic Action of PTH

PTH acts through dual signaling pathways in bone cells, with the osteoblast being the principal target. In the osteoblast, the Type I PTH/PTHrP receptor is coupled to both the adenylyl cyclase activating G protein, G_s , and the phospholipase C-activating G_q protein (54,55). PTH requires the first two amino acids and some part of the 25–34 AA region to activate G_s , but a fragment as small as 28–32 AA can activate G_q (54,55). Most of the skeletal actions of PTH can be related to cAMP/protein kinase A activation. Although it is likely that the balance of these two systems determines the overall biologic effect of PTH, it is well established that PTH activation of adenylyl cyclase is essential for osteoblast function (54,56). On the other hand, although cAMP/PKA activates early genes such as c-fos, the PKC system has also been shown to be operative when intermittent use of PTH increases osteoblast activity (57,58). Overall, the activation profile of PTH in bone cells leads to induction of several growth factor genes including those for IGF-1, IGF-II, and TGF- β . In addition, IGFBP-1, -4, -5 are induced by PTH as are IGF binding proteases -3 and -5 (54,55,57–63). The net effect of PTH on IGF-1 production is limited rather exclusively to bone cells.

Intermittent exposure of PTH for 4–6 wk in ovariectomized animal models leads to increased cancellous thickness (but not trabecular number). Cancellous bone mass and strength are greater, even in the absence of estrogen. Microscopically, bone cell turnover is enhanced, reminiscent of pubertal bone expansion (J. Hock, personal communication). On a cellular level, PTH enhances recruitment of preosteoblasts from marrow stromal cells, and induces maturation of lining osteoblasts, both of which increase collagen synthesis (54,60). Expression of skeletal IGF-1, as noted, is markedly enhanced, *in situ*, and *in vitro* by PTH administration (59,62,63). PTH induces the synthesis of osteoblastic cytokines such as IL-6, which when secreted, target early osteoclasts for recruitment. This process allows for coupling of bone resorption to formation although *in vivo* data suggest that bone formation indices increase consistently more and earlier than bone resorption markers. Thus the balance favors new bone formation, giving rise to the anabolic properties of intermittent PTH.

Notwithstanding these observations, the underlying molecular physiology accounting for the true anabolic effect of PTH remains unknown. In addition, it is uncertain why intermittent, low dose PTH administration differs so dramatically in its effect on bone cells from chronic sustained PTH treatment in which catabolic effects at cortical sites predominate (58). Recently, evidence has emerged that PTH reduces osteoblastic apoptosis, prolonging osteoblast survival and possibly potentiating its differentiated function in collagen synthesis (64).

hPTH has been used as the intact 1–84 amino acid molecule and as fragments (65,66). Other peptide fragments that have received attention include hPTH (1–38) (67) and PTH (1–31) (49).

OBSERVATIONAL STUDIES OF PARATHYROID HORMONE (TABLE 1)

Small, uncontrolled observational trials employed PTH alone, followed by trials with other agents. More recent controlled clinical trials with PTH are reviewed in the next section. In 1976, Reeve et al. treated four osteoporotic women with 6 mo of hPTH (1–34) at 100 mg/d and found an acceleration of bone turnover, with bone formation outweighing resorption (68). Slovik et al. administered hPTH (1–34) to six osteoporotic patients, 450 or 750 U/d, for 3–4 wk. The higher dose seemed to cause a net breakdown of bone (increased urinary calcium excretion without an increase in intestinal calcium absorption) (69). A larger study involved 21 patients (16 women and 5 men) with osteoporotic fractures (some of whom were on other drugs, such as vitamin D, estrogen and testosterone). The patients were treated with hPTH (1–34) 400–500 U/d for 6–24 mo. Radiocalcium kinetic studies showed bone formation and skeletal mass to be increased. These findings correlated with increases in trabecular bone volume by analysis of iliac crest biopsies. However, there was no improvement in overall calcium balance, thought to be possibly due to a loss of cortical bone (70), a result seen in seven of the patients who showed a downward trend in femoral neck bone mass (71). It was thought that calcium balance did not improve because PTH was not adequately stimulating 1,25-dihydroxyvitamin D formation (a concern in elderly patients with osteoporosis [72]). This led to subsequent protocols in which PTH was used in combination with other agents.

In the hopes of improving calcium absorption (73,74), PTH was next used in combination with 1,25-dihydroxyvitamin D. Eight osteoporotic men were given hPTH (1–34)

Table 1
Observational Trials

<i>Patient # and sex</i>	<i>Duration of treatment</i>	<i>PTH fragment, dose, regimen</i>	<i>Coadministered agents</i>	<i>Trial design</i>	<i>Results</i>	<i>Ref.</i>
4 women	6 mo	(1–34) 100 mcg/d	None	Observational	Accelerated bone turnover, with formation greater than resorption	(68)
5 women, 1 man	3–4 wk	(1–34) 450 or 750 U/d	None	Each patient their own control	Improved calcium balance with the lower dose	(69)
16 women, 5 men	6–24 mo	(1–34) 400–500 U/d	None	Observational	⁴⁷ Ca studies showed a 70% increase in cancellous bone volume; bone loss seen in distal femur	(70)
8 men	1 yr	(1–34) 400–500 U/d	1,25(OH) ₂ D ₃ 0.25 mcg/d	Observational	QCT showed 98% increase in vertebral BMD; no change in radial shaft BMD (by SPA); increases in calcium absorption	(73)
30 women	1–2 yr	(1–34) 400–500 U/d	1,25(OH) ₂ D ₃ 0.25 mcg/d	Control group (only given calcium)	Increase in vertebral BMD by QCT (32%) and DPA (12%); decreased BMD at cortical site (5.7%)	(74)
11 women, 1 man	1 yr	(1–34) 500 U/d	Estrogen or nandrolone	Comparison	Vertebral BMD increased 50% by QCT; no cortical loss. Biopsies showed cancellous bone growth	(75–77)
11 women, 1 man	64 wk	(1–34) 1500 U/d for 1 wk out of four, for 16 cycles	1,25(OH) ₂ D ₃ 0.25 mcg/d for 3 wk out of four, for 16 cycles	Observational	Decrease in activation frequency; histology revealed reductions in formation and resorption surfaces	(78)
6 women, 2 men	90 wk	(1–38) 400 U/d for 2 wk out of 15, for 6 cycles	Etidronate 5 mg/kg for 2 wk out of 15, for 6 cycles	Observational	No increase in bone mass	(79)

2 women, 6 men	14 mo	(1–38) 720–750 U/d sequentially	Calcitonin intranasally 200 U/d sequentially	Control group (on other treatments)	Vertebral BMD increased 15% by QCT; no change in forearm BMD by SPA	(80)
17 women, 3 men	200 d	(1–38) 400 U/d for 14 d out of 100 d	Half received calcitonin 100 U/d for 8 wk after PTH	Control group (only got PTH)	PTH alone: vertebral BMD increased 13% by DPA, radial bone density decreased 11% by SPA. PTH + calcitonin: no change in BMD	(81,82)
20 women	90 d	24-h (1–34) infusion once, then 800 U/d for 28 d	Half received calcitonin 75 U/d for 42 d	Control group (only got PTH)	Bone formation markers increased during daily PTH injections; histology confirmed increased bone formation. Calcitonin did not have a significant effect.	(42)
220 women	48 wk	(1–34) 50, 100, or 200 U/wk	None	Randomized, double-blind	Dose-dependent increase in BMD lumbar spine; 6.9% at highest dose. No significant change in hip BMD. Slight increase in total body BMD.	(83)

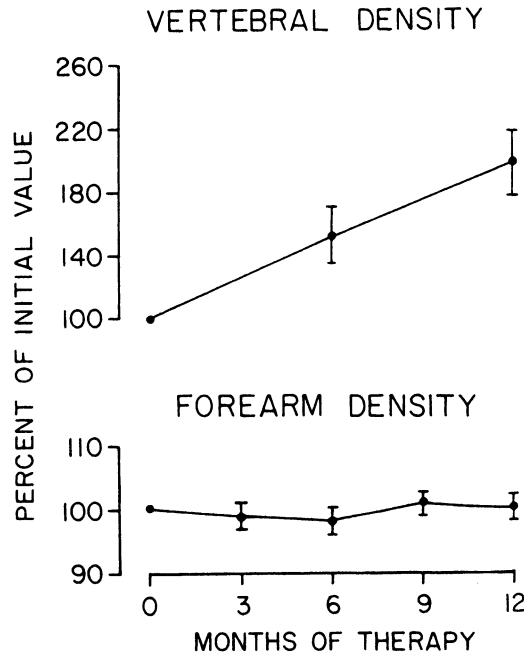


Fig. 4. Bone mineral density measured in osteoporotic men given PTH and 1,25-dihydroxyvitamin D. Each point represents the mean \pm SEM for 12 vertebrae in 4 men (trabecular bone) or 8 radii in 8 men (cortical bone). Taken with permission from ref. 73.

along with oral 1,25-dihydroxyvitamin D by Slovik et al. (73). In four subjects who had vertebral bone density measured by quantitative computed tomography, there was a remarkable two-fold increase in bone mineral density over one year, with an increase in calcium absorption and no loss of radial bone (Fig. 4) (73). In a similar study, 30 women with osteoporosis were given either daily hPTH (1–34) with calcitriol and calcium, or calcium only, for 1–2 yr. Spine bone mineral density increased in the women given PTH by 12% by dual photon absorptiometry and by 32% by quantitative computed tomography (QCT), while there was no change in the control group. The greater increase seen with QCT is consistent with the increases being mainly in cancellous bone. However, the increase in spine bone mineral density seemed to plateau after 6–12 mo. Cortical bone density measured by single photon absorptiometry (SPA) decreased by 5.7% with PTH, as compared to a 1.7% decrease with calcium only (74). It is possible, however, that the cortical loss was exaggerated, reflecting the lag in bone mineralization from the PTH-induced bone turnover (48).

In these early studies, PTH was also administered together with antiresorptive agents in small, observational trials. The antiresorptives (sex steroids, bisphosphonates, calcitonin) were used to minimize the expected cortical bone loss from PTH. hPTH (1–34) was studied in 12 patients with either estrogen or nandrolone for 8 of 12 mo (75). The PTH with estrogen group had an increase of 50% by QCT. There was no evidence of cortical bone loss (75). Radioisotopic measures of bone formation showed increases (76) and calcium balance improved. Biopsies of the PTH and estrogen group showed increments in cancellous bone volume, wall width and trabecular plate width with a decrease in trabecular spacing (76,77).

Other small studies involved PTH in combination with agents in a cyclical pattern. Short-term PTH was expected to activate a cohort of remodeling units, followed by a progressive sequence of depressed resorption, free formation, repeated stimulation (15).

These “ADFR” protocols yielded mixed results. In one small study, PTH and vitamin D were given sequentially to twelve osteoporotic patients, 11 women and 1 man. The subjects were treated with high doses of daily hPTH (1–34) for 1 wk, followed by three weeks of treatment with 1,25-dihydroxyvitamin D; this was repeated for sixteen cycles. Mean calcium balance increased, but there was an overall decrease in bone turnover and activation frequency. Histomorphometric analysis of bone biopsy showed reductions in resorption and formation surfaces (78).

In another study of sequential therapy, PTH and etidronate were administered in a cyclical pattern to eight patients with osteoporosis (some of whom had been previously treated with varied agents). Patients were given hPTH (1–38), because of its presumed greater potency (67) for 2 wk. During the second week, etidronate was added for 2 wk. This cycle was repeated six times, with 12-wk drug-free intervals in between. Calcium balance improved in four patients and decreased in two patients, but there was no increase in bone mass (79).

A third sequential study, with PTH and calcitonin, gave more promising results. Eight osteoporotic patients were treated with hPTH (1–38) at relatively high doses (720–750 U/d) in a sequential pattern for 32 wk of the year and intranasal calcitonin for 24 wk of the year. Vertebral bone density increased 15% by QCT (as compared to a “control” group, who were on other treatments) and forearm bone density did not change by single photon absorptiometry (in both patients and “controls”) (80).

In yet another sequential study with PTH and calcitonin (81,82), 20 patients (17 women and 3 men) received hPTH (1–38) daily for 2 wk (after an initial 24-h PTH infusion). Half then received calcitonin for 8 wk, followed by a 30-d drug-free period before repeating the cycle. After two cycles, in the PTH-only group, bone density increased by 13% (by dual photon absorptiometry [DPA]), while forearm bone mineral content (BMC) (by SPA) decreased by 11%. In the PTH+calcitonin group, there were no changes in bone density at either site. Since all subjects received PTH, it is unclear whether the cortical decrease was from PTH, or other factors, such as estrogen deficiency (82).

A trial of PTH given alone in postmenopausal osteoporotic women used a weekly regimen of PTH (83). The study involved 220 patients with osteoporosis who were given weekly injections of hPTH (1–34) of either 50, 100, or 200 U over 48 wk. There was no placebo group. The lumbar spine bone density increased in a dose-dependent fashion (0.6, 3.6, and 8.1%, respectively). A densitometric measurement on the second metacarpal did not change significantly. Bone alkaline phosphatase activity increased initially in all three groups, then fell to below baseline levels by wk 48. Urinary crosslinks decreased in the 50- and 20-U group, but not in the 100-U group. Interestingly, the investigators found a reduction of symptomatic back pain in 30–40% of each group, although it was not statistically significant (83).

CONTROLLED CLINICAL TRIALS (TABLE 2)

More recently, larger, randomized, placebo-controlled clinical trials have been performed with PTH alone and in combination with other agents. In general, the results show that PTH causes a marked increase in vertebral bone density, a more modest increase in hip bone density and no change or only a slight decrease in radial bone density. Bone markers seem to show an uncoupling of bone turnover in favor of formation, as evidenced by an initial increase in formation markers, followed by an increase in resorption markers.

Table 2
Controlled Clinical Trials

<i>Patient # and sex</i>	<i>Duration of treatment</i>	<i>PTH fragment, dose, regimen</i>	<i>Supplements</i>	<i>Coadministered agents</i>	<i>Trial design</i>	<i>Results</i>	<i>Ref.</i>
40 women	1 yr	(1–34) 400 mcg/d	Calcium 1200 mg/d	Nafarelin 200 mcg intranasally bid	Randomized, placebo-controlled	Spine BMD increased (7.5%), no change in FN or cortical bone. 1 yr after withdrawal BMD continued to increase at all sites	(84,86)
217 women	1 yr	(1–84) placebo, 50, 75, or 100 mcg/d	Calcium 500 mg/d Vit D 400 U/d Vit A 5000 U/d	None	Randomized, placebo-controlled, double-blind	Dose-dependent increase in BMD lumbar spine; 6.9% at highest dose. No significant change in hip BMD. Slight decrease in total body BMD	(102)
23 men	18 mo	(1–34) 400 U/d	Calcium 1200–1500 mg/d Vit D 400–800 U/d, 6 mo before and during study	None	Randomized, placebo-controlled, double-blind	Lumbar spine increase of 13.5%; FN increase of 2.9%. Distal 1/3 of radius had minimal decline.	(91)
34 women	3 yr	(1–34) 400 U/25 mcg/d	Calcium 1500 mg/d	Premarin 0.625 or Estraderm 50 mcg/d	Randomized, placebo-controlled (not blinded)	13% increase in spine BMD, 2.7% increase in hip BMD, no loss of cortical bone; reduction in loss of vertebral height	(92)
74 women	2 yr	(1–34) 400U/d	Calcium 1500 mg/d Vit D 800 U/d	Premarin 0.625, medroxy-progesterone, if needed	Randomized, double-blind, placebo-controlled	29% increase in vertebral BMD; 11% increase in FN BMD	(94)

51 women	1 yr	(1–34) 400 U/d	Calcium 1000 mg/d Vit D 800 U/d	Premarin 0.625 mg/d, Prednisone 5.0–20 mg/d	Randomized, placebo- controlled (not blinded)	Vertebral BMD increased (96,97) by 11% DXA, 35% QCT, hip density increased by 2%. 1 yr after treatment: vertebral BMD increase by 13% DXA, 46% QCT, hip density increased by 5%. Slight decrease in radial density in both groups.
30 women	2 yr	(1–34) 800 U/d for 1 mo out of 3	Calcium 500 mg/d	Calcitonin for 6 wk out of 3 mo	Randomized	No benefit from addition of calcitonin. PTH alone: spine BMD increased by 10.2%; PTH+calcitonin, increased by 7.8%. Suggestion of PTH alone improving FN BMD. Cortical BMD not measured.
66 women	1 yr PTH 1 yr alendronate	(1–84) at 50, 75, or 100 mcg/d	Calcium 500 mg/d Vit D 400 U/d	Alendronate 10 mg/d	Randomized, double- blind; first year placebo -controlled	At end of second year: (87) vertebral BMD increased up to 14.6%; trend of improved hip BMD; whole body BMD improved (up to 6.1%) after a prior decrease at the end of the first year.
1637 women	21 mo	(1–34) at 20 or 40 mcg/d	Calcium 1000 mg/d Vit D 400– 1200 U/d	None	Randomized, double- blind, placebo- controlled	Vertebral fracture risk (88) reduced by 65%; nonvertebral fracture risk reduced by 54%

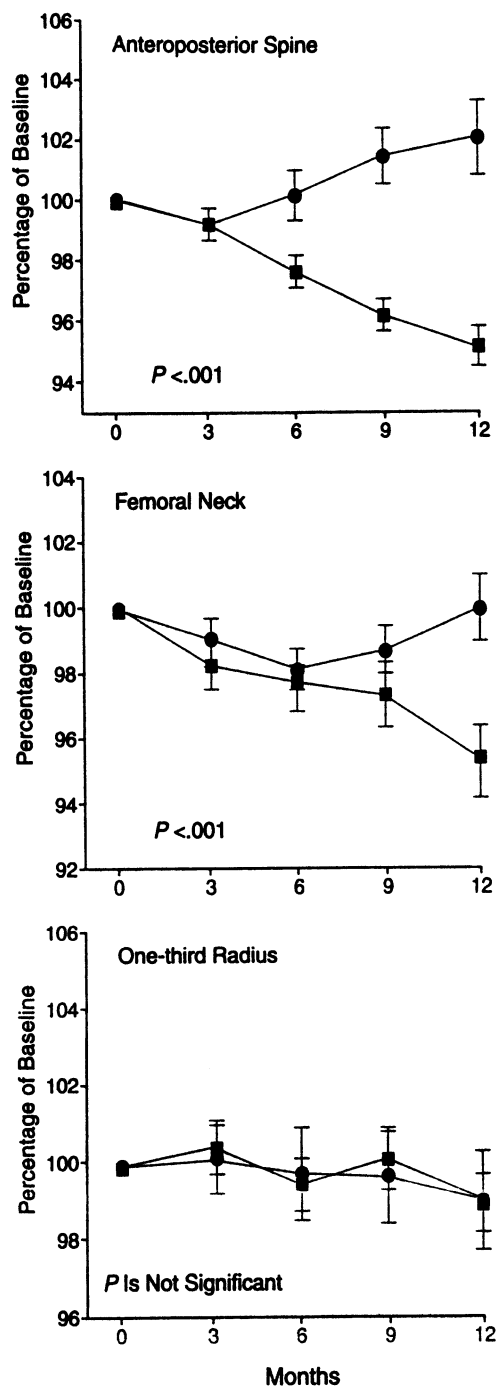


Fig. 5. The effect of PTH in women treated with nafarelin for endometriosis. Bone mineral density of the lumbar spine measured in the anteroposterior and lateral projections, femoral neck, trochanter, one-third radius and total body in women receiving nafarelin alone (squares) or nafarelin plus human parathyroid hormone (circles). Values are expressed as the percentage of baseline (SEM). The P values are for the comparisons of changes at 12 mo between the two groups as assessed by student's t-test. The error bars for some measures in the women treated with nafarelin alone are contained within the symbols. Adapted with permission from ref. 85.

PTH ADMINISTERED ALONE

PTH in Estrogen-Deficiency

PTH alone was administered to 40 young women with endometriosis, for one year (84–86). The women were rendered estrogen-deficient for treatment of their endometriosis with nafarelin, a long-acting GnRH analog; the dose was targeted to lower estrogen to postmenopausal levels. The women were randomized to hPTH (1–34) 40 mcg injected daily or not for 1 yr. At 6 mo, the nafarelin-only group had a decrease in lumbar spine density (3.5% in the lateral projection and 2.8% in the anteroposterior projection); at 12 mo the reduction in bone mineral density was even greater (4.9% in both projections). The nafarelin+PTH group, in contrast, showed an increase in lumbar spine bone density at 6 mo (3.4% in the lateral projection and no change in the anteroposterior projection) and even more so at 12 mo (7.5% in the lateral projection and 2.1% in the anteroposterior projection) (Fig. 5).

Femoral neck bone density decreased slightly and similarly in both groups at 6 mo. At 12 mo, however, the nafarelin-only group had fallen further to 4.7%, while the nafarelin + PTH group returned to baseline levels. Cortical bone loss did not seem to be significant in the nafarelin + PTH group. Throughout the treatment period, there was no change in either group of radial bone density, while total body bone density decreased 2% without PTH and was unchanged with PTH.

Bone markers of formation (alkaline phosphatase and osteocalcin) and resorption (urinary hydroxyproline and pyridinolines) increased in both groups, but more so in the nafarelin + PTH group. In the presence of PTH, these markers peaked at 6–9 mo and then began to decline. At 12 mo, despite the reduction from peak levels, bone markers were still substantially above the placebo group.

Finkelstein et al. extended observations for a post-treatment year to determine whether discontinuance of PTH is associated with further gains in bone mineral density or a return toward baseline values (86). In both groups, the GnRH analog was stopped during this additional year of the study. Although both groups had an expected increase in bone density after the GnRH-analog was stopped, the women in the nafarelin-only group did not return to their baseline, pretreatment bone densities. One year later, they were still significantly below their baseline values at the anteroposterior spine and femoral neck, and tended to be lower than their baseline values at the trochanter and total body. Conversely, in the nafarelin + PTH group, bone density was significantly above their original baseline values at the anteroposterior and lateral spine and was similar to their baseline values at other sites (Fig. 6). Bone density thus continued to improve in the nafarelin + PTH group after PTH was withdrawn. These observations are similar to the increases in bone density seen after parathyroidectomy for primary hyperparathyroidism. The withdrawal of PTH seemed to permit remineralization of the bone remodeling space which had been enlarged due to PTH therapy (50,53). Thus, two mechanisms of bone gain may be operative in the context of PTH therapy. When PTH is present, gains appear to be due to a direct anabolic effect on bone formation. When it is withdrawn, the return of bone dynamics to normal permits remineralization of the remodeling space much in the manner of antiresorptive therapy.

PTH in Postmenopausal Osteoporosis

PTH as single therapy has also been studied in postmenopausal osteoporotic women. One RPCT was a larger multicenter phase II dose finding with 1–84 PTH in 207 post-

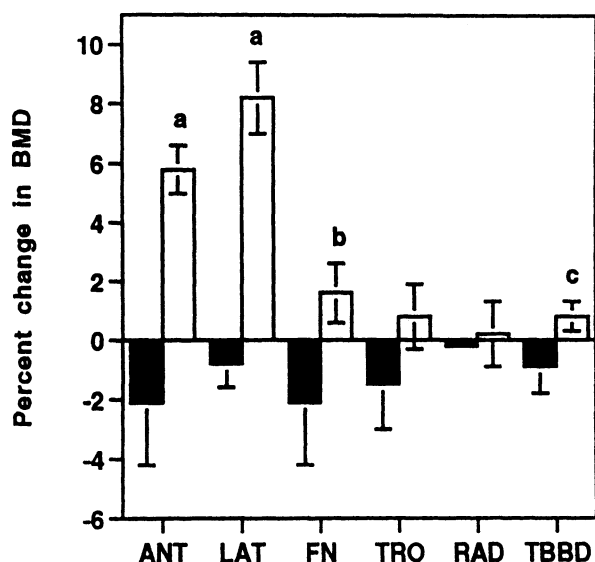


Fig. 6. The effect of PTH withdrawal in women treated with nafarelin for endometriosis. Percent changes in BMD of the lumbar spine measured in the AP (ANT) and lateral (LAT) projections, femoral neck (FN), trochanter (TRO), one third radius (RAD), and total body (TBBD) from the baseline evaluation until the 1-yr follow-up examination in the patients treated with nafarelin alone (group 1; solid bars) and in the women receiving nafarelin plus hPTH (1–34) (group 2; open bars). Values are expressed as the mean \pm SEM. P values are for the between-group difference in percent change in BMD. a, $P < 0.001$; b, $P = 0.005$; c, $P = 0.037$. Reproduced with permission from ref. 86.

menopausal women with low bone mineral density (T scores < -2.0) (87). After 1 yr, women receiving the highest dose, 100 mg (400 IU) PTH, demonstrated a nearly 8% increase ($p < 0.0001$) in spine bone mineral density with virtually no change in femoral bone mineral density and a slight decrease in total body bone mineral density (Fig. 7) (85). Lower doses of PTH showed lesser changes in spine bone mineral density, consistent with a dose dependent effect on trabecular bone mineral density. PTH treatment was not associated with any major adverse events, although nearly 20% of the subjects receiving the highest dose of PTH did have transient hypercalcemia.

The second and largest RPCT to date tested daily administration of 20 or 40 μ g of subcutaneous 1–34 hPTH in 1637 women with postmenopausal osteoporosis (i.e., low bone mineral density and fractures) (88). Median follow-up was 21 mo and for the two doses of PTH spine bone mineral density increased between 12–15%. Femoral bone mineral density also increased significantly. Most impressive, however, was the relative risk reduction for both vertebral and nonvertebral fractures in those women receiving either 20 or 40 μ g of PTH: RR for vertebral fracture (20 μ g) = 0.35 (0.22–0.55), 40 mg: 0.31 (0.19–0.50), and RR for nonvertebral fracture = 0.46 (0.25–0.88) for either dose of PTH. Nausea and headache were the most common side effects, and these occurred infrequently and in a dose dependent manner. Less than 5% of the women had sustained increases in serum calcium above the normal range in any of the treatment groups.

PTH in Osteoporotic Men

Idiopathic osteoporosis in men constitutes a group for whom PTH could be ideally suited in view of the fact that this is a disorder of low bone turnover (89,90). Twenty-three men with

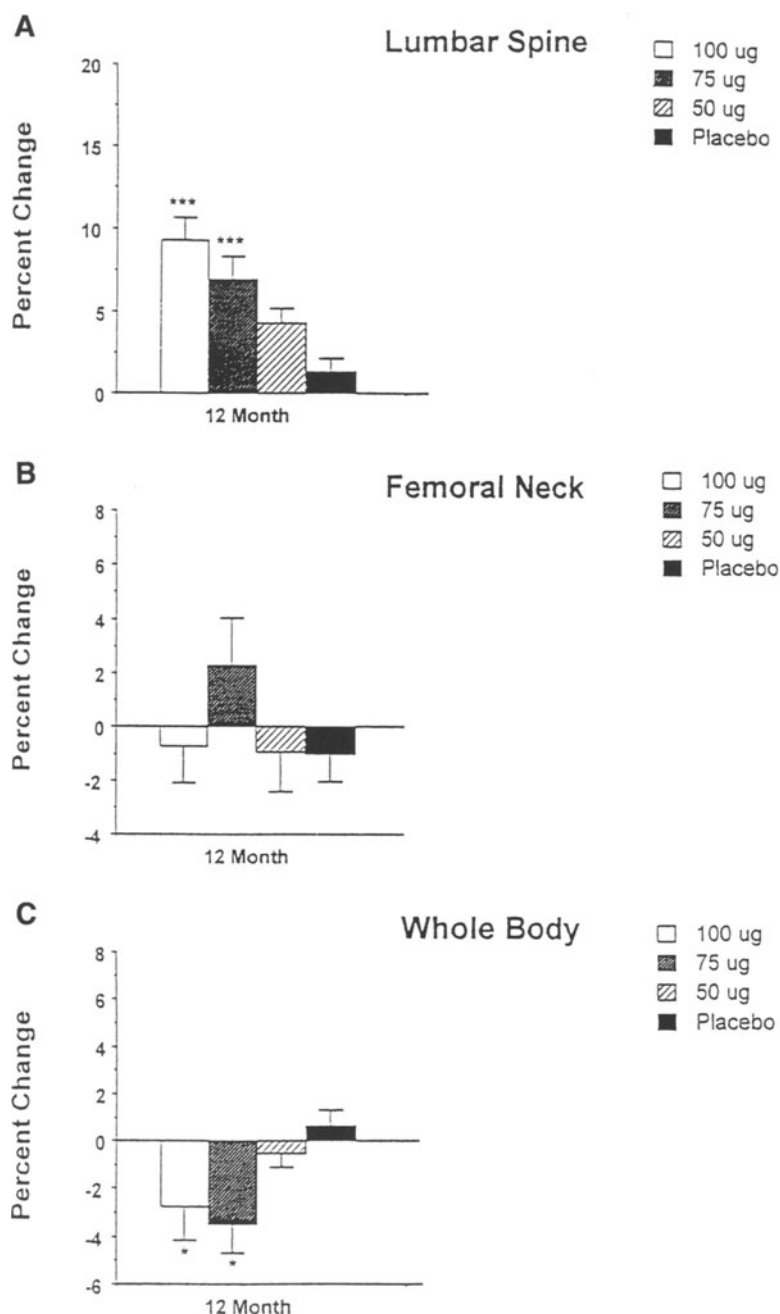


Fig. 7. PTH monotherapy in postmenopausal women with osteoporosis. BMD (percent change from baseline; mean \pm SEM) in postmenopausal women given 100, 75, or 50 μ g PTH or placebo for 1 yr. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (compared to placebo group). Adapted with permission from ref. 7.

idiopathic osteoporosis were randomized to hPTH (1–34) 400 U/d or placebo in an 18-mo clinical trial published by Kurland et al. (91). The PTH group had an impressive increase of lumbar spine bone density of 13.5% with an increase of femoral neck density 2.9% and only

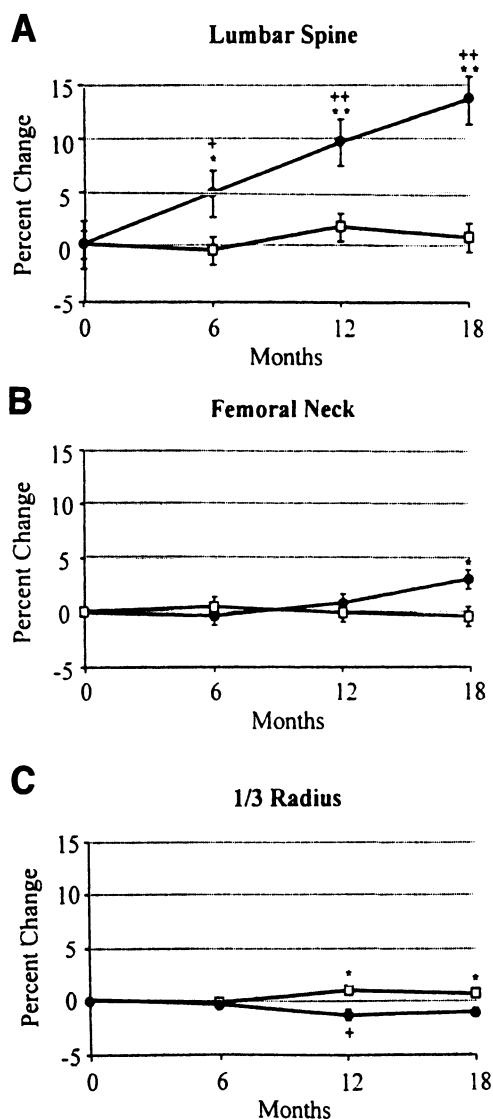


Fig. 8. Changes in bone density after PTH (1-34) treatment in men with idiopathic osteoporosis. Bone density at lumbar spine (A), femoral neck (B), and 1/3 site of the distal radius (C) in men receiving PTH (●) and in controls (□). The data are shown as percent changes from baseline \pm SEM for lumbar spine, FN, and 1/3 radius. * $P < 0.05$ for repeated measures analysis of between-group comparisons. ** $P < 0.005$ for repeated measures analysis of between-group comparisons. +, $P < 0.05$ for repeated measures analysis of within-group comparisons between baseline and 6, 12, or 18 mo. ++, $P < 0.005$ for repeated measures analysis of within-group comparisons between baseline and 6, 12, or 18 mo. Taken with permission from ref. 91.

a minimal decrease in the distal 1/3 of the radius (Fig. 8). Bone markers of formation and resorption increased in the PTH group and predicted changes in bone mass (91).

PTH IN COMBINATION WITH ANOTHER AGENT

PTH has been studied in controlled trials in conjunction with antiresorptive agents. The rationale for this combination is that PTH should stimulate bone formation, while

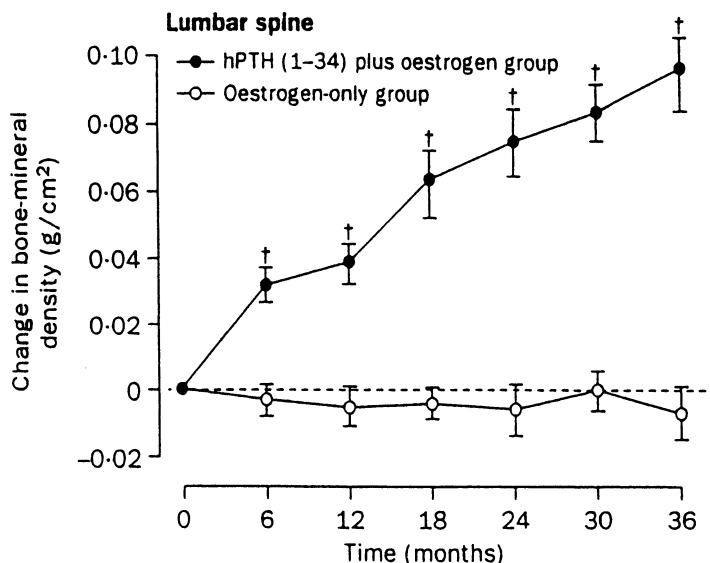


Fig. 9. The effect of PTH in women being treated with estrogen. Changes in lumbar spine bone mass when estrogen was given with or without PTH over 3 yr to postmenopausal osteoporotic women. Taken with permission from ref. 92.

the antiresorptive agent should limit any catabolic effect as well as contribute its own effect to increase bone mineral density. In concept, therefore, anabolic and antiresorptive therapy should be more effective than either approach alone.

PTH and Estrogen

PTH combined with estrogen was studied in a three year randomized controlled trial of 34 postmenopausal osteoporotic women taking hormone replacement therapy, with or without hPTH (1-34) daily (92). The PTH group had significant increases in bone density: 13% at the spine (the greatest increase occurring during the first year of treatment), 2.7% at the hip and 7.8% in the total body (Fig. 9). There was no evidence of cortical bone loss. PTH was associated with a reduction in loss of vertebral height (i.e., vertebral fractures). Bone formation markers (osteocalcin) rose before bone resorption markers (N-telopeptide) during the first 6 mo, followed by a return of both to baseline values.

A subgroup of subjects were assessed for any long-term adverse effect on calcium homeostasis; i.e., whether there would be endogenous PTH suppression after exogenous PTH treatment (93). Ten subjects were given hypocalcemic challenges before, during and after the study and were found to have normal endogenous PTH responsiveness to the hypocalcemic challenge.

In a more recent study by Roe et al., 74 postmenopausal women were randomized to receive either 400 IU of PTH (1-34) or placebo while remaining on stable doses of conjugated equine estrogens (94). There was a nearly 30% increase in spine bone mineral density, as well as an 11% increase in femoral bone mineral density as measured by DXA among women receiving combination therapy compared to those on estrogen alone. The increase in vertebral bone mineral density was even greater, close to 80%, when measured by QCT of the vertebrae. Taken together these data make two important points: 1) PTH plus estrogen has a greater effect on bone mass than either alone; 2) the beneficial

effects of combination therapy are found in both the spine and in the femur, the two most vulnerable areas for subsequent fractures.

PTH and Estrogen in Glucocorticoid-Induced Osteoporosis

The bone-forming effects of PTH could be particularly suited for steroid-induced osteoporosis, since steroids suppress bone formation (95). Lane et al. conducted a 12-mo, randomized, controlled trial of 51 postmenopausal women on hormone replacement therapy and steroids, who were randomized to hPTH (1–34) for one year or not (placebo injections were not used). In the PTH group, bone density increased 35% by QCT and 11% by DXA. The total hip bone density increased by 2% in the PTH group, while the forearm density decreased by ~1% in both groups. Bone markers showed an increase of bone formation in the first 3 mo, while resorption peaked at 6 mo (96). This is indicative of an early uncoupling of bone turnover, in favor of formation, similar to that seen in a previous study of PTH and estrogen (92).

The subjects were subsequently studied 12 mo after PTH withdrawal (97), as were the young women who had been given the PTH and GnRH analog (86). Bone density continued to increase after PTH was stopped. Two years after the start of treatment, the PTH group had further increases in vertebral bone density (cumulative change: 45.9% by QCT and 12.6% by DXA) and total hip and femoral neck bone density (cumulative change: 4.7 and 5.2%, respectively). The estrogen-only group, in comparison, did not have any significant changes their spine or hip bone density (Fig. 10). Both groups had a slight decrease of forearm bone density (1.5%) over the 2-yr period. Bone markers returned to baseline within 6 mo after stopping PTH. The further increases in bone density in this protocol after PTH was discontinued follows the pattern seen after parathyroidectomy for hyperparathyroidism and the protocol using nafarelin as previously described.

PTH with Calcitonin

PTH was studied in a randomized trial with calcitonin (98), in addition to earlier, smaller trials (42,80–82). Similar to the rationale for other antiresorptive agents, calcitonin use was hoped to limit the resorption induced by PTH and thus augment the overall anabolic effect. But calcitonin combined with PTH did not prove to have an additive effect (98). The study patients were 30 postmenopausal osteoporotic women who were all given high dose hPTH (1–34); half were given calcitonin. The drugs were administered sequentially: PTH was given for one month out of three and the calcitonin was given for 6 wk out of the 3-mo cycle. A subgroup of 20 patients from this study had bone biopsies done after only 28 d of PTH injections (42), which showed increases of bone formation.

At the end of 2 yr, however, the difference between the two groups was not significant. The PTH-alone group had a vertebral density increase of 10.2% and the PTH+calcitonin group had an increase of 7.9% (Fig. 11). There was a difference in the femoral neck between the two groups (PTH-alone increased 2.4%, while PTH + calcitonin decreased 1.8%), but this was also not significant.

Both groups had significant increases of bone formation markers during the PTH injections of the first year, which fell slightly during the second year.

PTH with Alendronate

PTH has been studied in combination with alendronate (87,99,100). The rationale for adding alendronate is to decrease the enlarged remodeling space created by the PTH and

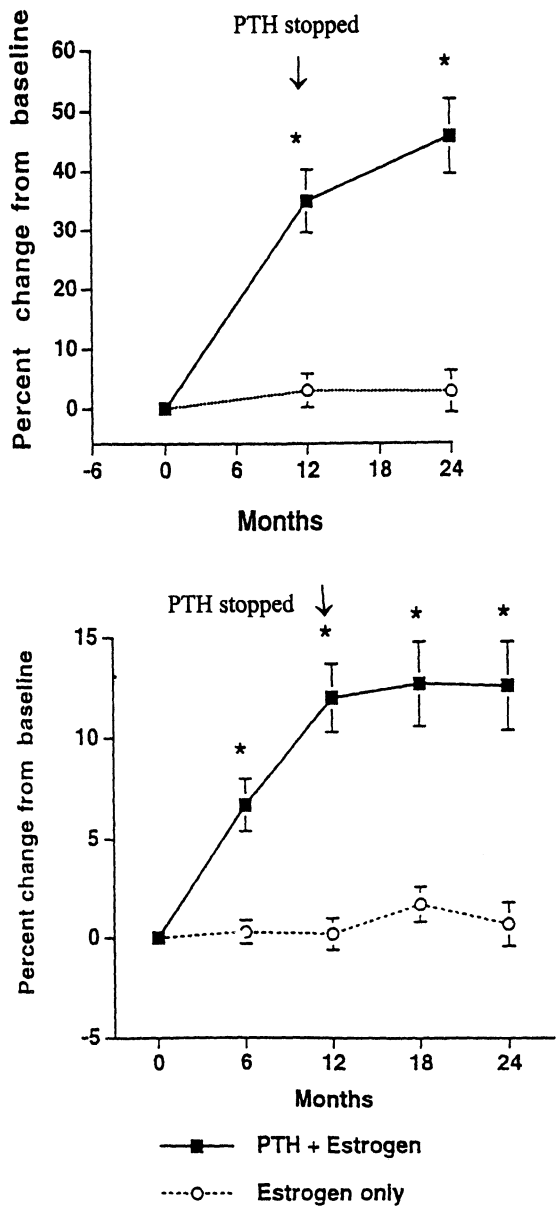


Fig. 10. The effect of PTH to counteract bone loss due to glucocorticoids. Changes in lumbar spine BMD when estrogen was given with or without PTH for 12 mo to postmenopausal women on steroids. Mean percent change (\pm SEM) from baseline measured by QCT (top) and DXA (bottom). * $p < 0.001$ between groups at 12 and 24 mo. Adapted with permission from ref. 97.

thus consolidate the gains in bone density and prevent any decline. A different bisphosphonate, clodronate, was not able to maintain the improved bone density gained with intermittent PTH in an early study (99). Another more encouraging, small study evaluated 10 osteoporotic women already on alendronate; half were given daily PTH for 6 wk as well (100). The PTH + alendronate group had an increase in markers of formation within 3 wk, which returned to baseline once PTH was stopped. This seemed to indicate that PTH can stimulate bone formation even in the presence of a bisphosphonate (likely *de novo*, since there was no preceding stimulation of resorption).

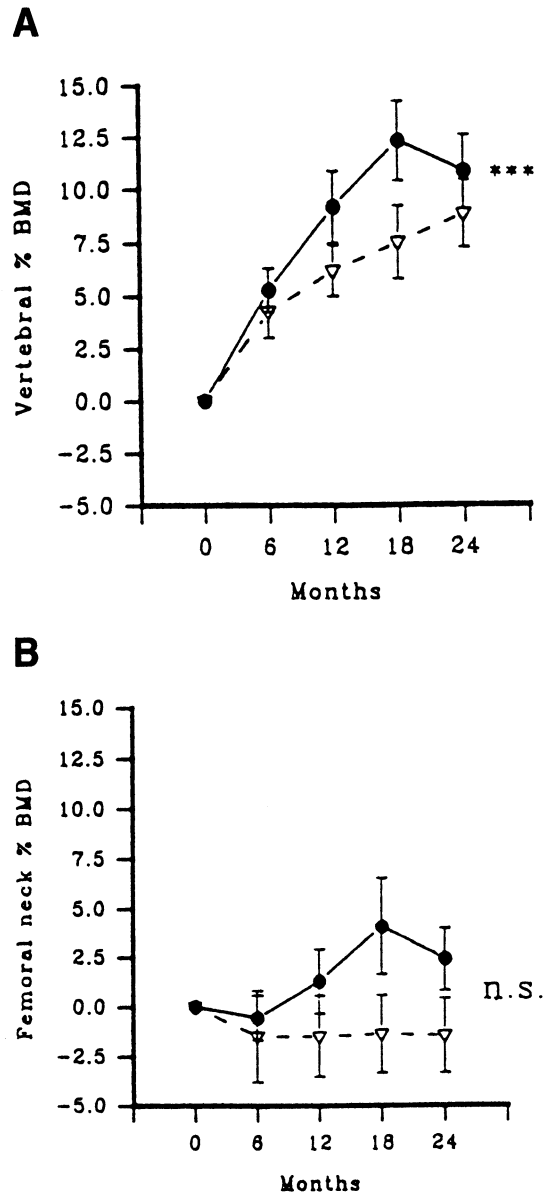


Fig. 11. Combination therapy of PTH and calcitonin. Percent changes in BMD (mean \pm SEM) over the lumbar spine (**A**) and femoral neck (**B**) for patients treated with cyclical PTH alone (●) and treated with PTH plus calcitonin (▽). ***, $P < 0.001$ across time; no difference in final outcome between groups. n.s., No significant difference at baseline, across time or between groups. Taken with permission from ref. 98.

More recently, a randomized controlled trial was performed to assess the effects of PTH followed by alendronate (87). Sixty-six women with postmenopausal osteoporosis were treated for 1 yr with either placebo or varying doses of hPTH, followed by one year of alendronate for all subjects. For the first year, subjects were randomized to placebo, 50, 75, or 100 mcg of hPTH (1–84). During the PTH-phase, vertebral bone density increased in a dose-dependent fashion: 6.9 and 9.2% at the two highest doses. After the year of alendronate treatment, those who had received the highest dose of PTH had

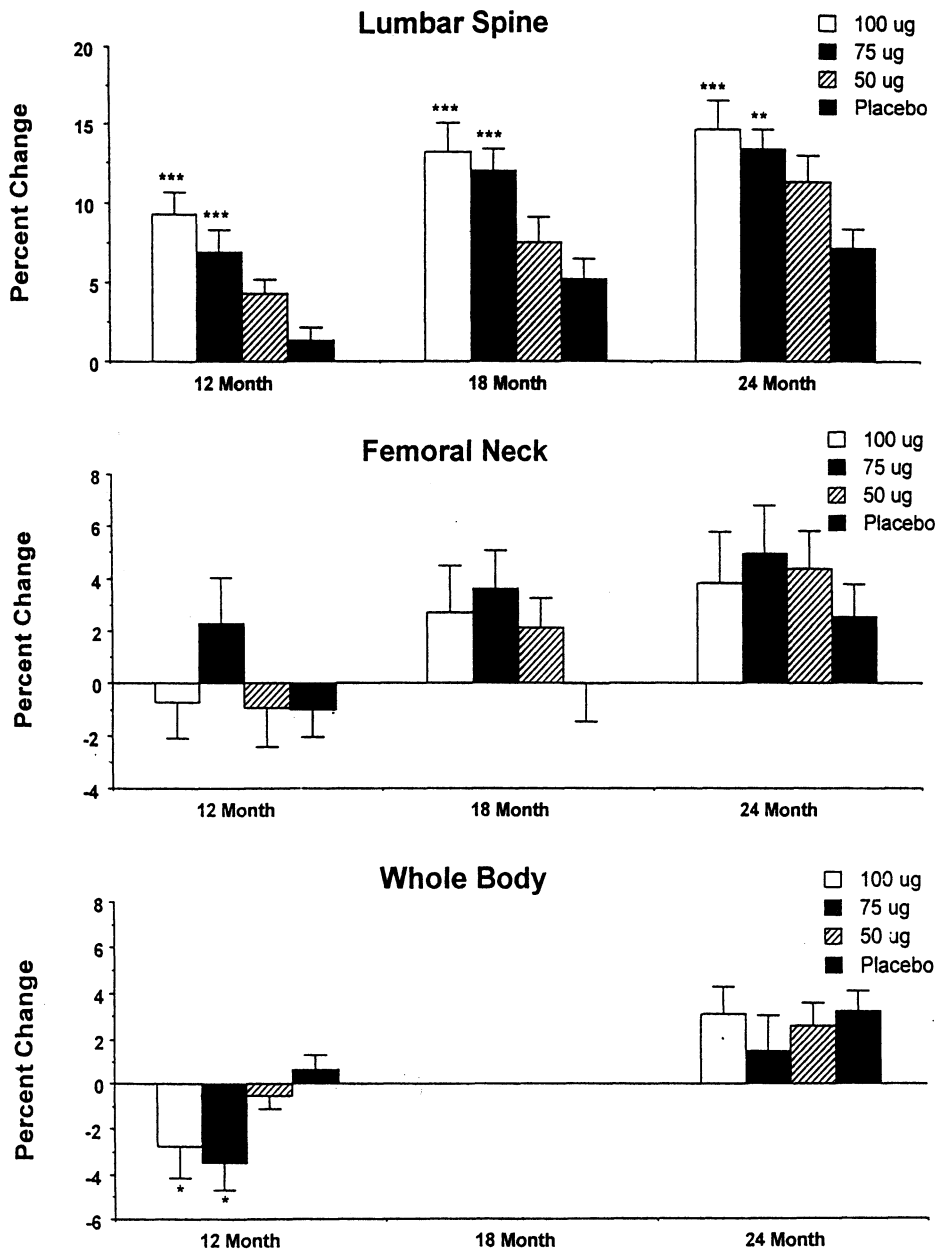


Fig. 12. Sequential therapy with PTH followed by alendronate. BMD (percent change from baseline; mean \pm SEM) in postmenopausal women given 100, 75, or 50 mg PTH or placebo during the first year followed by 10 mg alendronate daily during the second year. Month 12 data represent the changes observed after 1 yr of PTH or placebo. Month 18 and 24 data represent the changes observed after an additional 6 and 12 mo of alendronate treatment, respectively. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (compared to placebo/alendronate group). Taken with permission from ref. 87.

impressive spinal bone density increases of up to 14.6%. There was a trend toward increased hip bone density that was not statistically significant. Total body bone density initially decreased at the end of the PTH year (by 3.5 and 2.8% in the two highest doses), but improved by the end of the alendronate year (Fig. 12). Bone markers of both

formation and resorption significantly increased in all three treatment groups during treatment with PTH and decreased to below baseline values after one year of alendronate.

The addition of alendronate thus seemed to halt, or reverse, loss of cortical bone and resulted in further improvement in trabecular bone at the spine. It is unclear, however, how much of the bone density improvement happened solely as a result of a continued anabolic effect after PTH withdrawal, since there was no placebo group which did not receive alendronate. What is not known, additionally, is whether PTH and a bisphosphonate used simultaneously is better, worse or no different than sequential therapy. A randomized trial sponsored by the NIH is currently underway to test that hypothesis.

SAFETY OF PTH

There are important concerns about the use of PTH as an anabolic agent in osteoporosis. More fractures could theoretically occur as a result of the increased bone turnover and remodeling space. In addition, along with the increase in cancellous bone mass, there is the fear of cortical bone loss, or a "cortical steal" phenomenon (101). This could potentially increase hip fractures, an important source of morbidity and mortality, since the hip is composed of both cancellous and cortical bone. However, preliminary histomorphometric analysis has not revealed a loss of cortical bone. Bone biopsy analyses of 16 osteoporotic patients treated with PTH were recently presented by Dempster et al. (103). In eight osteoporotic men (treated for 18 mo) and eight osteoporotic women on estrogen (treated for 36 mo), PTH increased cortical width along with connectivity density and maintained cancellous bone mass.

PTH appears to be a safe drug. In most studies, hypercalcemia and hypercalciuria are monitored and if necessary, the PTH dose is decreased. In the dose of PTH expected to become available, this is unlikely to be a significant problem. In some human studies, anti-PTH antibodies were found in a few subjects, although their presence did not seem to attenuate the effects of PTH.

Long term studies (18–24 mo) with hPTH (1–34) administered to 6-wk-old Fisher 344 rats have demonstrated an increased risk of osteogenic sarcoma. This effect, which is dose dependent, does appear to be related to duration of use, and would be consistent with lifetime exposure in a growing animal to an anabolic agent which increases osteoblast proliferation. Other studies (6–12 mo) in primates have failed to find an association between intermittent administration of PTH and osteogenic sarcoma. Moreover, there have been no documented cases of osteogenic sarcoma in patients with primary, secondary or tertiary hyperparathyroidism from several large patient cohorts. Although further safety data are needed, it is reasonable to assume that PTH is safe in humans for short-term administration to those most likely to benefit; i.e., postmenopausal women and men with clinical fractures and low bone mineral density.

CONCLUSIONS

PTH is a promising anabolic therapy for osteoporosis. Clear evidence in human trials now documents the ability of PTH to stimulate cancellous bone formation; new evidence shows an associated decrease in fractures. However, there are still unanswered questions about PTH. More large-scale studies are needed to further determine the fracture rate with PTH and the effects of PTH withdrawal. Furthermore, trabecular bone formation seems to plateau after 12–18 mo of PTH treatment, raising the possibility of a resistance to PTH treatment over time, and concepts to design protocols to use PTH as intermittent therapy.

In the future, PTH is likely to be modified for easier and more targeted delivery. Other delivery systems such as oral, transdermal, or inhaler may become available. Ultimately, when the anabolic and catabolic mechanisms of PTH can be clearly distinguished from a mechanistic and molecular view, it may be possible to develop PTH analogs that are purely anabolic.

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The Cellular and Molecular Aspects of Immunosuppressant Osteoporosis

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INTRODUCTION

The advent of immunosuppressant agents that prevent organ rejection and prolong life has revolutionized the field of organ transplantation. However, these drugs possess numerous side effects, including bone loss that results in osteoporosis and fracture. This chapter will focus on the mechanism of action of the major immunosuppressants used clinically. These include glucocorticoids, the calcineurin inhibitors (cyclosporin A [CsA] and tacrolimus [FK506]), rapamycin, and several others, such as azathiaprine and mycophenolate mofetil (MMF). We will describe in vitro and in vivo studies that have elucidated their action on bone.

GLUCOCORTICOIDS

Effects on the Skeleton

Glucocorticoids are known to cause osteoporosis (1). It is estimated that as many as 50% of patients treated with glucocorticoids will suffer fractures (2). Both cortical and trabecular (cancellous) bone is lost, trabecular bone being more commonly affected. Thus, sites such as the ribs, vertebrae, and distal long bones are high-risk sites for fractures (2). In addition to fractures, osteonecrosis or avascular necrosis is common with glucocorticoid treatment and causes collapse of the femoral head in as many as 25%

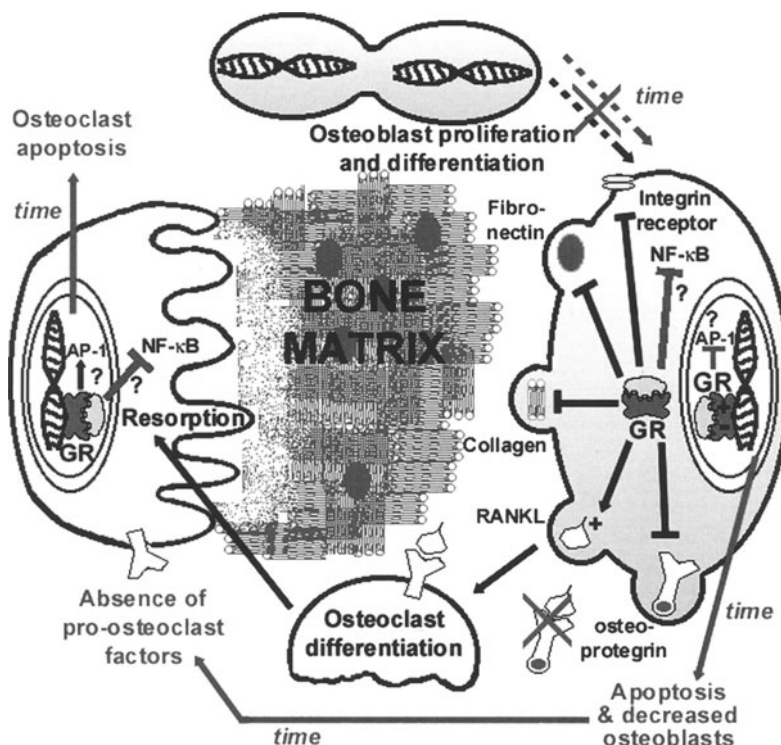


Fig. 1. Glucocorticoids have temporal-dependent effects on bone. By reducing the production of integrin receptors, fibronectin, and collagen, they initially inhibit bone formation (black arrows, right side). Glucocorticoids also initially induce bone resorption by increasing pro-osteoclastic factors, such as RANK-L. Over time (purple arrows), osteoblast numbers continue to decrease with osteoclast numbers following; this may be due to either decreased proliferation or increased apoptosis.

of patients (3). The incidence and severity of the osteoporosis caused by glucocorticoids is a direct function of the dosage used and the length of treatment, with the greatest bone loss occurring during the first 6–12 mo of therapy (4). Postmenopausal women appear to be at the highest risk (5). However, glucocorticoid-induced osteoporosis is partially reversible when therapy is stopped (2).

Ultimately, glucocorticoids produce a low turnover osteoporosis (6,7). Initially, osteoblast activity and proliferation is decreased, while osteoclastic activity is increased (see Fig. 1) (8,9). This phase is characterized by an increase in the number and activity of resorption surfaces with resulting reductions in bone volume. As a result of decreased osteoblast activity, there is a decrease in the total amount of bone replaced in each remodeling cycle (7). Over time, bone formation continues to be decreased and resorption falls to levels at or below normal, with resorption cavities lacking both osteoclasts and osteoblasts (8) (Fig. 1).

Modulation of Hormone Effects by Glucocorticoids

The development of glucocorticoid-induced osteoporosis is mechanistically complex and involves effects on calcium, parathyroid hormone (PTH) and sex steroid metabolism. Glucocorticoids inhibit calcium transport across cell membranes causing

hypercalciuria and reduced intestinal calcium absorption (10) and thus a net negative calcium balance. The increase in renal calcium excretion is initially due to a rapid decrease in bone formation and early increases in resorption, but in patients on long-term glucocorticoid therapy, the fasting hypercalciuria is due to both increased mobilization of calcium from the skeleton and a reduction in renal tubular reabsorption of calcium (11,12). These alterations in calcium metabolism can lead to secondary hyperparathyroidism (1). Additionally, glucocorticoids induce hypogonadism by inhibiting pituitary gonadotropin secretion (13,14). Moreover, adrenocorticotrophic hormone (ACTH) suppression and adrenal atrophy, due to exogenous glucocorticoid therapy, can result in low levels of adrenal androgens (1). Finally, glucocorticoids inhibit growth hormone secretion in normal men (15). It is interesting to note, however, that serum levels of growth hormone and IGF-1 are normal in patients receiving glucocorticoids (16).

Cellular Actions of Glucocorticoids

Glucocorticoids directly inhibit osteoblast function and result in decreased replication, differentiation and life span of these cells (17). Osteoblast and osteocyte apoptosis is increased in mice and humans receiving glucocorticoids. Studies of glucocorticoid-induced osteonecrosis revealed apoptotic osteocytes and cells lining cancellous bone (17).

Most of the effects of glucocorticoids on osteoblastic activity and differentiation result from reduced transcription of collagen, fibronectin, osteonectin, osteocalcin and other genes (9,18). Glucocorticoids inhibit the synthesis of fibronectin (19), an important molecule involved in cell adhesion, and decrease the expression of β_1 integrins in organ culture (20,21). β_1 integrins belong to a family of transmembrane receptors that mediate cell adhesion to extracellular matrix macromolecules (such as fibronectin and type-1 collagen), and modulate cell migration and differentiation (20). A decrease in both fibronectin and β_1 integrin synthesis may contribute to the pathogenesis of glucocorticoid-induced osteoporosis (*see* Fig. 1). How do glucocorticoids mediate these effects in osteoblasts? A possible answer comes from data in osteoblast cultures—Cbfa-1 expression is suppressed upon glucocorticoid application (22). The transcription factor core-binding factor A1 (Cbfa-1) is responsible for differentiation of progenitor cells into osteoblasts (23).

Glucocorticoids have several indirect actions on the osteoblast. They promote the bone-forming action of PGE₂ (24,25) by interfering with arachidonic acid release and inhibiting the expression of prostaglandin G/H synthase-2 (PGHS-2) in cultured mouse calvariae (26). Although the implications are unclear, glucocorticoids increase the expression of IGFBP-1 and IGFBP-6 and decrease the expression of IGFBP-3, 4, and 5 in osteoblast cultures (27,28). Note that an IGFBP can inhibit or enhance IGF-1 action (27,29).

Glucocorticoids increase the sensitivity of osteoblasts to PTH and 1,25-dihydroxy-vitamin D, both of which are known to inhibit collagen synthesis (30). PTH increases osteoblast cyclic adenosine monophosphate (cAMP) production in the presence of glucocorticoids (31,32). Glucocorticoids affect myocytes causing muscle wasting and decreased muscle strength (so-called glucocorticoid-induced myopathy) and may thus ultimately result in decreased bone formation (33).

There is some evidence that glucocorticoids directly stimulate osteoclastic activity. Transient increases in bone resorption after glucocorticoid administration are thought to result from enhanced osteoclast number and activity (34). Osteoblasts may play a role in the initiation of osteoclastic bone resorption through the modulation of the

osteoprotegerin system. In osteoblast and stromal cell cultures, glucocorticoids increase production of receptor activator of NF κ B ligand (RANKL), a pro-osteoclastic differentiation factor, as well as decrease production of osteoprotegerin (OPG), an anti-osteoclastic differentiation factor (35,36). This may be responsible for the initial increase in resorption (*see* Fig. 1). Over time, however, a decrease in the number of osteoclasts occurs likely because of a combination of reduced osteoclast differentiation and increased apoptosis. Indirectly, the gradual diminution of osteoblasts (*see* section on osteoblasts above) likely also leads to a reduction in pro-osteoclastic factors (e.g., RANKL) thereby inhibiting osteoclastic proliferation and differentiation. This hypothesis may explain the temporal sequence of initial increase in osteoclastic activity, followed by a reduction in the number of active osteoclasts (*see* Fig. 1).

Molecular Pathways for Glucocorticoid Action

Glucocorticoids diffuse passively through cell membranes and bind to specific glucocorticoid receptors (GRs) in the cytoplasm and nucleus. This activated receptor complex is able to alter transcription by two opposing transcriptional mechanisms (37). It may bind to glucocorticoid-response elements (GREs) that act as enhancers and stimulate transcription (38). Binding to negative GREs (nGREs) displaces transcription factors and represses transcription (39,40).

An example of negative transcriptional regulation occurs in the human osteocalcin promoter (41). Here, the nGRE overlaps the TATA box (42). Decreased collagen synthesis is another example of glucocorticoid-induced negative transcriptional regulation (1,2,43). Although differences between species exist, the human pro α 1 (I) collagen promoter has several nGREs that may cooperatively repress transcription, with further modulation occurring at the transforming growth factor- β (TGF- β) site (43). Regulation of collagen expression is probably complex, and decreased production of TGF- β , prostaglandin E₂ (PGE₂) and insulin-like growth factors (IGFs) likely alter collagen synthesis (30,44). Glucocorticoids also increase the synthesis and production of collagenase, an action that may compound the negative effect on collagen production.

Apart from transcriptional effects mediated through interactions of GR with DNA, GRs can bind to transcription factors and cofactors without binding to DNA (45). In this case, positive or negative transcriptional regulation by the GR depends upon the composition of the transcription factor complex. In thymocytes, for example, interaction of GRs to *c-Jun* homodimers causes stimulation of AP-1 mediated transcription (46). In contrast, the interaction of the GR with *c-Jun/c-Fos* heterodimers causes repression of transcription (46–48). The GR can also bind to CREB, STAT3, STAT5, and NF- κ B (49). Additional effects include suppression of the synthesis of NF- κ B and AP-1 (48,50), and enhancement of I κ B (51).

Glucocorticoids also may regulate cell proliferation by cell cycle actions. For example, the GR is phosphorylated by cyclin-dependent kinases (CDKs) and MAP kinases (52). The greatest phosphorylation occurs in G₂/M phase (53), although the precise role of this effect in osteoblast regulation is unclear.

In osteoclasts, glucocorticoids also appear to modulate transcriptional regulators. GRs are known to inhibit NF- κ B activation (49) and this may represent a way by which osteoclast proliferation and differentiation is inhibited. However, this relationship may be complex. For example, multiple cytokines including RANKL are known to induce NF- κ B activation in osteoclasts and may overpower repression by the GR, in part explaining the initial stimulation of osteoclastic activity by glucocorticoids.

Glucocorticoids, may directly stimulate osteoclast apoptosis. Although the expression of glucocorticoid receptors in osteoclasts is still debated (54,55), there is some evidence that apoptosis of osteoclasts is dependent on GR activation. For example, a GR antagonist, RU-486, prevents glucocorticoid induced osteoclast apoptosis (56). In thymocytes, the induction of apoptosis likely involves gene transcription, as it requires ATP and protein synthesis (57,58). Apoptosis may result when interference occurs with transcription factors necessary for survival. The apoptotic process may occur through the “mitochondrial” pathway; it is reversed in thymocytes by the expression of Bcl-2, Bcl-X_L, Apaf-1, and caspase-9 (49).

CYCLOSPORIN

Cyclosporin A (CsA) is a cyclic endecapeptide, initially isolated from the fungus *Tolypocladium inflatum* Gams (59). It is used in the treatment of rheumatoid arthritis (60), psoriasis, nephrotic syndrome, and inflammatory bowel disease (61), but its most recognized role is in the prevention of organ rejection after transplantation (62–67). CsA causes bone loss in humans (62–68). Elucidating CsA's effect on bone mineral metabolism has been difficult because of confounding factors such as the underlying disease and other drugs used in combination with CsA, including glucocorticoids (69,70).

CsA induces high turnover osteoporosis in several animal models. Both formation and resorption parameters are increased, but resorption exceeds formation (71–74). The osteoporosis is severe and is dose- and duration-dependent (71). Bone histomorphometry shows decreased percent trabecular bone volume, increased osteoclast number and increased bone formation. Increased serum osteocalcin and urinary N-telopeptide levels indicate an increase in bone formation and resorption, respectively (71). CsA also increases serum 1,25-(OH)₂D levels by directly increasing 1 α -hydroxylase activity in rats (75). Rat experiments have also shown that CsA enhances estrogen deficiency-induced bone resorption in vivo (72).

The effect of CsA is reversible. When CsA is withdrawn, normalization of most histomorphometric parameters, with the exception of a reduced bone volume, occurs within 2 wk (73). CsA-induced osteoporosis is ameliorated, or even reversed, by the administration of PGE₂, 1,25-(OH)₂D₃, salmon calcitonin, 2-pyridinyl ethlidene bisphosphonates (2-PEBP), alendronate, 17 β estradiol in estrogen deficiency and raloxifene (70,76–79). Growth factors and cytokines that fail to reverse CsA-induced bone loss in the rat include TGF β , endothelin and IL-6.

CsA-induced osteoporosis appears to be mediated by the presence of T lymphocytes, as CsA does not produce osteopenia in the T cell depleted nude rat (80). However, in vitro, we have observed osteoclast inhibition by CsA, rather than the expected stimulation. Furthermore, the CsA-induced osteoclastic inhibition was not reversed at 24 or 48 h in the presence of T-lymphocytes. The molecular basis of this discrepancy is unclear. It is possible that the osteoblast (not present to an appreciable extent in vitro) may also mediate the CsA effect.

CsA increases the expression of IL-1, an osteoclastogenic cytokine (81). It also inhibits IFN- γ , GM-CSF, and IL-2 production from lymphocytes (82). Specifically, CsA prevents the activation of NF-AT and thus cytokine transcription (82). However, the actions of CsA in T-cells are more complex than an inhibition of the synthesis of cytokines. For example, CsA indirectly reduces the action of IL-2 by increasing the expression of TGF- β , a known inhibitor of IL-2-stimulated T cell proliferation (83). In addition, CsA causes partial inhibi-

tion of IL-2 cell surface receptor expression, thus affecting the autocrine and paracrine effects of IL-2 on the T cell (84).

CsA is lipophilic and gains access to the cytoplasm by passive diffusion (61). Within cells, CsA binds to its intracellular protein targets: the cyclophilin (CyP) family of proteins (84,85). This CsA-CyP complex has several actions, including the initiation of gene transcription and the regulation of protein folding. CsA-CyP first inhibits calcineurin activity. Calcineurin (protein phosphatase 2B), a ubiquitous calcium/calmodulin-dependent protein phosphatase (86), is found in neural tissues, T and B cells, as well as osteoclasts (87). The induction of certain cytokines such as IFN- γ , GM-CSF, and IL-8 are dependent upon the calcineurin pathway, and are therefore blocked by CsA (82).

It was hypothesized that calcineurin inhibition is the molecular mechanism for immunosuppressant-induced bone loss and it was observed that mice lacking the most abundant calcineurin isoform, A α , showed a reduction, up to 50%, in bone formation (unpublished). This was associated with reduction in cortical width, but modest reductions in trabecular bone. Moreover, osteoblast maturation was stimulated directly by calcineurin A α , consistent with the effect of calcineurin A α gene deletion on bone formation, and calcineurin upregulated key transduction and effector molecules, including the transcription regulators, NFAT1c and I κ B, and the type 1 isoform of the ryanodine receptor, a Ca²⁺ release channel. Calcineurin was also found to dephosphorylate I κ B β , the NF κ B inhibitor, suggesting that I κ B may be an alternative calcineurin substrate.

The CsA-CyP complex regulates gene transcription by altering certain nuclear proteins, such as NF-AT, AP-3, and NF- κ B (88,89). Specifically, it has been shown that CsA-CyP prevents the dephosphorylation of NF-AT, probably through calcineurin inhibition (82). CyPs may also directly regulate gene transcription by interacting with RNA polymerase II (90) and this action may regulate genes important for cell-cycle progression through the G₂/M phase. However, the effect of CsA on this pathway remains to be established. Additionally, CyPs have been speculated to bind to and activate steroid receptors (GR and ER) through heat shock protein-90 (91,92). CsA inhibits this binding and activation.

CsA binds competitively to the N-terminal portion of CyPs and inhibits *cis-trans*-peptidylprolyl isomerase (PPIase) activity (93). PPIases catalyze the rotation about the peptide bond proceeding proline, that can be rate limiting for the folding of new proteins. PPIases also regulate the activity of mature proteins by promoting the assembly or transport of subunits. In yeast, none of the CyPs are necessary for survival (94), and it remains to be established if CsA inhibits the folding of cell specific proteins that could play a role in osteoporosis.

TACROLIMUS (FK506)

Tacrolimus (FK-506, fujimycin) is a potent lipophilic macrolide immunosuppressant produced by *Streptomyces tsukubaensis* (95,96). It is a more potent immunosuppressant than CsA and has been used effectively in organ transplantation in both humans and animals (95,97). Tacrolimus has been shown to be associated with trabecular bone loss in cardiac transplant patients (98). In rats, tacrolimus causes a reduction in percent trabecular area with increased measures of both bone formation and bone resorption (99). However, resorption exceeds formation, and net bone loss occurs. The bone loss induced by tacrolimus is equal or greater than those with CsA and GCs (99).

While tacrolimus and CsA are chemically unrelated and have different cytosolic binding sites (100), they have similar effects at the molecular level (95). Tacrolimus

binds to its own class of immunophilins called FK Binding Proteins (FKBPs). Four forms of FKBP have been isolated (84); None of them are essential for growth in yeast (94). FKBPs have PPIase activity that is inhibited by tacrolimus (84,101). Like the CsA-CyP complex, the tacrolimus-FKBP complex inhibits calcineurin (84,102,103) and inhibition of IL-2, 3, 4, and IFN- γ has been observed in T-cells in response to tacrolimus in vitro (101). Also like CyPs, FKBPs can bind to and activate steroid receptors with *hsp*-90; this binding is likely inhibited by tacrolimus (91,92).

While the mechanisms of action of CsA and tacrolimus seem to be similar, the changes in mineral metabolism induced by the two drugs may not be identical. Lower serum calcium concentrations and a resultant increase in serum PTH levels was seen in tacrolimus treated rats, but not in CsA treated rats. Also, CsA increases serum levels of bone gla protein (BGP), but tacrolimus-treated rats have normal or only mildly increased levels (99,104). A possible mechanism for these differences may be an effect of tacrolimus' actions on ryanodine (RyR)/Ca²⁺ release channels. The traditional Ca²⁺ release channel is composed of four RyR proteins and four FKBPs, with PKA phosphorylation leading to FKBP dissociation and channel inactivity (105). In neuroblastoma cells tacrolimus enhances caffeine-induced Ca²⁺ release, an effect presumably mediated by the RyR (106). Tacrolimus does not affect IP₃-induced Ca²⁺ release, but does inhibit extracellular Ca²⁺ uptake. Several RyR isoforms, specifically type 2, exist in osteoclasts and are important in regulating resorption (107,108). Notably, the type-2 RyR regulates extracellular Ca²⁺ sensing and the propagation of Ca²⁺ signals in osteoclasts. Thus, the effect of tacrolimus' action on FKBPs and the resulting activation of RyRs might account for the difference seen between tacrolimus and CsA. This must be shown experimentally, however.

OTHER IMMUNOSUPPRESSANTS

Rapamycin

Rapamycin (sirolimus), a macrocyclic lactone, is a recently developed immunosuppressant that is structurally similar to FK506. Rapamycin exerts immunosuppressive effects by blocking signaling distal to IL-2 receptor activation, thereby suppressing T cell mediated immunity (109,110).

Rapamycin binds FKBPs and FKBP-rapamycin-associated protein (FRAP or RAFT) to inhibit the activation of p70 S6 kinase, and subsequently T-cell activation (111). In vitro, rapamycin has been shown to enhance osteoblast differentiation, (increased alkaline phosphatase (ALP) activity and osteopontin mRNA expression) while it reduces proliferation (112). The molecular mechanism may be similar to that of FK506 (i.e., FKBP binding), but the precise pathways are yet unclear. At immunosuppressive doses rapamycin has no effects on bone in vivo. However, in large doses, rapamycin produces hypogonadism in male rats that in turn result in severe osteopenia and fractures (113). When combined with low dose CsA, rapamycin is bone-sparing in the rat model.

Mycophenolate Mofetil

Mycophenolate mofetil has been licensed in the United States as an immunosuppressant for use in organ transplantation. Histomorphometric examinations of rats treated with oral mycophenolate mofetil for 28 d revealed no evidence of bone loss (114). This drug is rapidly replacing azathioprine in triple therapy with glucocorticoids and CsA.

Azathioprine

Azathioprine is a purine analog that interferes with DNA synthesis and produces its cytostatic effect on lymphoid cells (115). Azathioprine, used alone, seems to be relatively bone sparing. It does, however, lower serum osteocalcin levels (i.e., suppresses osteoblastic differentiation), an effect of unclear significance (115). Clinically, no untoward effects on bone mineral metabolism have yet been reported, but this may be a result of its limited clinical usage in situations other than transplantation.

Methotrexate

Methotrexate causes osteoporosis in humans (116). Methotrexate osteopathy is an uncommon clinical condition that arises from long-term oral maintenance therapy of methotrexate for childhood neoplasms such as acute lymphocytic leukemia (ALL) (117,118). Although children who are given methotrexate continue to grow normally, methotrexate may induce increased bone resorption and decreased bone formation (119–121).

Cell culture and rat studies show that methotrexate can affect osteoblasts (120). Short-term high-dose administration profoundly inhibits osteoblast *function* in rats, as evidenced by reductions in osteoid volume and thickness, but osteoblast numbers are maintained (121). Another study using long-term low-dose therapy found that bone formation was decreased, while bone resorption increased (119).

The mechanism of action of methotrexate is its competitive inhibition of folic acid reductase. This reduces the conversion of dihydrofolate to tetrahydrofolate and decreases the deoxyribonucleotide (dNTP) pool, thus impairing tissue-cell (including bone marrow) reproduction and repair (122,123). Whether this mechanism underlies the osteoporosis induced by methotrexate, or whether there are other yet uncharacterized cellular actions, is presently unclear.

Deoxyspergualin

15-Deoxyspergualin (heptanamide), was initially developed for its antibiotic and antitumor activity. In animal experiments it has also proven to be an effective and potent immunosuppressant (124). Possible immunosuppressive mechanisms of 15-Deoxyspergualin include the inhibition of formation and differentiation of cytotoxic T-cells, binding to *hsps*, and increased expression of various cytokines, including IL-2, IL-3, IL-4, IL-10, and IFN- γ (125). Information about the actions of 15-Deoxyspergualin on bone is sparse at best. The skeletal effects of other immunosuppressants such as mizoribine, brequinar sodium, diflunomide and azaspirane are unknown.

CONCLUSION

This chapter has focused on the cellular and molecular aspects of immunosuppressant induced osteoporosis. Three immunosuppressants were considered in detail. Glucocorticoids induce low turnover osteoporosis via a series of cellular effects. Through multiple mechanisms, they initially induce bone resorption while also inhibiting formation. Over time, osteoblast numbers continue to decrease followed by falls in osteoclast numbers and a decrease in the frequency of bone remodeling. Cyclosporin and tacrolimus both induce high turnover osteoporosis characterized by increased bone resorption. How they induce osteoporosis remains largely unknown, but likely involves modulation of calcineurin-dependent gene transcription, inhibition of peptidyl-prolyl isomerase activity,

and regulation of T-cell cytokine production. Other immunosuppressants induce osteoporosis via various mechanisms, including alterations in DNA synthesis (methotrexate and azathioprine), inducing osteoblast differentiation at the expense of proliferation (rapamycin), and increasing the expression of resorptive cytokines (deoxyspergualin).

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INTRODUCTION

The introduction of cyclosporine to transplantation immunology in the early 1980s resulted in marked improvement in short-term graft and patient survival and ushered in a new era for patients with end-stage renal, hepatic, cardiac, pulmonary and hematopoietic disease. The addition of cyclosporine, and later tacrolimus, to post-transplantation immunosuppression regimens permitted the use of lower doses of glucocorticoids. Therefore, it was initially expected that glucocorticoid-induced osteoporosis would be less of a problem in the cyclosporine era. During the past decade, however, it has become clear that organ transplant recipients managed with cyclosporine (1,2), and probably tacrolimus (3,4), continue to sustain rapid bone loss and fragility fractures. Moreover, transplantation-related bone loss and fractures may become increasingly common as more patients continue to undergo organ transplantation each year and survival continues to improve. This review will summarize our current understanding of the effects of the most commonly prescribed immunosuppressive agents on bone and mineral metabolism. The epidemiology, natural history and pathogenesis of bone loss and fracture after various types of organ transplantation will be reviewed. Recommendations for prevention of the acute phase of bone loss after organ transplantation, and treatment of established osteoporosis in organ transplantation candidates and recipients will be summarized.

SKELETAL EFFECTS OF IMMUNOSUPPRESSIVE DRUGS

The Bone-Remodeling System

The impact of immunosuppressive drugs on the skeleton is mediated through their interactions with the bone-remodeling system, an orderly progression of events by which

bone cells remove old bone tissue and replace it with new. Transplantation osteoporosis, as with most adult metabolic bone diseases, is the result of alterations in the bone remodeling system. Thus, it is helpful to review the orderly sequence of events that constitutes normal bone remodeling, in order to understand the pathogenesis of transplantation osteoporosis.

The two main processes by which remodeling occurs are known as resorption and formation (5). Conceptually, these processes are somewhat akin to the repair of cracks and potholes that develop in surfaces of highways. Remodeling occurs on the surfaces of both cancellous and cortical bone. The first step is activation of macrophage precursors to form osteoclasts, giant multinucleated cells that excavate or resorb a cavity on the bone surface. Osteoclasts express receptors for calcitonin, prostaglandins, calcium and vitronectin (integrin $\alpha_1\beta_3$). In general, approx 0.05 mm^3 of bone tissue is resorbed by each osteoclast, leaving small resorption pits on the bone surface called Howship's lacunae. This process takes approx 2–3 wk. After a brief rest period known as the reversal phase, local mesenchymal bone marrow stem cells differentiate into osteoblasts that are attracted to the empty resorption pits. There they accumulate as clusters of plump cuboidal cells along the bone surface. Osteoblasts have two major functions. They produce the proteins, both collagenous and non-collagenous, that constitute the matrix of the newly formed bone. Osteoblasts are also responsible for calcification of the matrix or osteoid after an approx 10-d period during which the osteoid has matured. Osteoblasts express receptors for parathyroid hormone, estrogens, vitamin D₃, cell adhesion molecules (integrins) and several cytokines. The complete remodeling cycle at each remodeling site requires approx 3–6 mo. This process serves to replace old, micro-damaged bone with new, mechanically stronger bone.

In normal adults, bone remodeling results in no net change in bone mass. Bone loss develops in any situation in which bone remodeling becomes “uncoupled,” such that the rate of resorption exceeds the rate of formation. This most often occurs when the rate of resorption is so elevated that it is beyond the capacity of the osteoblasts to restore the original amount of bone volume. However, bone loss may also develop in the setting of depressed bone formation, such that even normal amounts of resorbed bone cannot be replaced. It is very likely that transplantation-related bone loss results from both a primary decrease in the rate of bone formation and a primary increase in the rate of resorption.

Glucocorticoids

Glucocorticoids, an integral component of most posttransplantation regimens, are notorious for causing osteoporosis. Prednisone or methylprednisolone are commonly prescribed in high doses (50–100 mg of prednisone or its equivalent daily) immediately after transplantation and during episodes of severe rejection, with gradual reduction over weeks to months. Total exposure varies with the organ transplanted, the number and management of rejection episodes, and the practice of individual transplantation programs.

Glucocorticoids cause immunosuppression by inhibiting both proliferation of T-cells and expression of certain cytokines. Since several of the cytokines that are suppressed by glucocorticoids (IL-1, IL-6, TNF- α , interferon- γ) have been found to stimulate bone resorption (6), it is thought that glucocorticoids must cause bone loss and fractures by mechanisms other than direct stimulation of bone resorption. These mechanisms, summarized in Table 1, include several direct and indirect effects on bone and mineral homeostasis (7,8). Direct effects include immediate and profound inhibition of bone

Table 1
Glucocorticoid Actions That Contribute to Bone Loss

Direct effects → Inhibition of bone formation

- Decrease osteoblast recruitment and differentiation
- Decrease osteoblast synthesis of type I collagen and osteocalcin
- Induce apoptosis of osteoblasts and osteocytes
- Inhibit growth hormone secretion
- Reduce production/activity of skeletal growth factors (IGF-1, PGE₂, TGF-β)

Indirect effects → Stimulation of bone resorption (controversial)

- Inhibit intestinal calcium absorption
 - Increase urinary calcium losses
 - Secondary hyperparathyroidism
 - Hypogonadotrophic hypogonadism
 - Decrease secretion of adrenal androgen and estrogen
-

formation by decreasing osteoblast recruitment and differentiation, synthesis of type I collagen, and induction of apoptosis of osteoblasts and osteocytes both in vitro and in vivo (9). These effects are reflected biochemically by low serum levels of osteocalcin (bone GLA protein), a major non-collagenous bone matrix protein secreted by osteoblasts. Indirect effects include inhibition of growth hormone secretion and decreased production or bioactivity of certain skeletal growth factors (IGF-1, PGE₂, and TGF-β), actions that also reduce bone formation.

Glucocorticoids may also increase bone resorption (10), although this continues to be far more controversial than their inhibitory effects on bone formation. The mechanism of their potential effects on bone resorption is likely related to impaired calcium transport across cell membranes, causing reduced intestinal calcium absorption and hypercalciuria, negative calcium balance and secondary hyperparathyroidism. Glucocorticoids also cause hypogonadotrophic hypogonadism and reduced secretion of adrenal androgens and estrogens, which may also be associated with increases in bone resorption. These contrasting effects of glucocorticoids upon bone formation (decreased) and resorption (increased) prevent osteoblasts from replacing the increased amount of bone resorbed at each remodeling site, and rapid bone loss ensues. Glucocorticoid-induced myopathy may also contribute to bone loss by altering gravitational forces on the skeleton, reducing weight-bearing activity and mobility, and to fracture rates by increasing the propensity for falls.

Virtually all patients taking glucocorticoids sustain significant bone loss (7), regardless of age, race, gender, or menopausal status. Postmenopausal, estrogen-deficient women may be at greater risk for fracture than other groups, because glucocorticoid-related bone loss is superimposed upon that already sustained because of aging and estrogen deficiency. In general, bone loss is most rapid during the first 12 mo and is directly related to dose and duration of therapy. Areas of the skeleton rich in cancellous bone (ribs, vertebrae, and distal ends of long bones) and the cortical rim of the vertebral body are most severely affected and also fracture most frequently.

In recent years, there has been a trend toward more rapid lowering of glucocorticoid doses after transplantation or rejection episodes, and an increase in the use of alternative drugs to treat rejection. In more recently transplanted patients who have received lower

doses of steroids, significant bone loss persists although it may be less rapid than previously documented (11). Moreover, it should be noted that even rather small doses of glucocorticoids are associated with increased fracture risk. A recent British study found that doses of prednisolone as low as 2.5 mg daily were associated with a significant 55% increase in the relative risk of spine fractures; doses between 2.5 and 7.5 mg daily were associated with a 2.6-fold increase in the risk of spine fracture and a 77% increase in the risk of hip fracture (12). Thus, even in those programs that have embraced the use of lower doses of glucocorticoids, there is still sufficient exposure in the initial year to cause significant bone loss.

Cyclosporines

Cyclosporine is a small fungal cyclic peptide. Its activity depends upon the formation of a heterodimer consisting of cyclosporine and its cycloplasmic receptor, cyclophilin. This cyclosporine-cyclophilin heterodimer then binds to calcineurin (13). The cyclosporine-cyclophilin-calcineurin complex functions as a phosphatase inhibitor that inhibits genes expressing interleukin (IL)-2, receptors for IL-2, and the protooncogenes *H-ras* and *c-myc* (14). In recent years, it has become clear that cyclosporine has effects on bone and mineral metabolism that could contribute to bone loss after organ transplantation (Table 2) (6). When administered to rats in doses comparable to or in excess of those used to prevent allograft rejection, cyclosporine consistently causes extremely rapid and severe cancellous bone loss (15,16). The dominant histological finding in animals treated with cyclosporine is a marked increase in bone resorption. In contrast to the skeletal effects of glucocorticoids, bone formation is increased in these animals, although the increase in formation is insufficiently large to compensate for the increase in resorption.

The effects of cyclosporine on the skeleton are very likely mediated via T lymphocytes. Evidence in support of this mechanism includes the observation that cyclosporine does not cause bone loss in the athymic nude T cell-deficient rat (17). Moreover, cyclosporine H, an analogue that neither inhibits T cell function nor has immunosuppressant properties, does not cause bone loss in the rat (18). Zahner and colleagues (19) have also observed that T cells mediate the stimulatory effect of cyclosporine A on osteoclast formation.

Cyclosporine may affect bone at the cellular level in other ways. Cyclosporine increases gene expression of bone-resorbing cytokines. Both IL-1 and IL-6 mRNA are increased in bone and bone marrow extracted from cyclosporine-treated rats (20). Gene expression of osteocalcin is also increased by cyclosporine (20). In addition, recent animal studies suggest that parathyroid hormone (PTH) may modify cyclosporine-induced bone loss, since parathyroidectomized rats lose less bone than intact animals when exposed to cyclosporine (21). Recently, the gene for calcineurin (and its isoforms), a protein that is integral to the immunosuppressive action of cyclosporine, has been identified in osteoclasts and extracted whole rat bone. However, the calcineurin gene is not altered by cyclosporine action in bone (22).

Given the marked increase in bone resorption consistently observed in histologic studies, it is not surprising that drugs that inhibit bone resorption prevent or attenuate cyclosporine-induced bone loss in the rat (6). Estrogen (23), the selective estrogen receptor modulator, raloxifene (24), calcitonin (25), and the bisphosphonate, alendronate (26), have all been shown to be effective in this regard. Similarly, 1,25 dihydroxyvitamin D (27) and prostaglandin E2 (28) also prevent bone loss in cyclosporine-treated rats. In contrast, testosterone (29) does not ameliorate bone loss in this model.

Table 2
Skeletal Effects of Cyclosporine (and Tacrolimus)^a

-
- Increase expression of bone resorbing cytokines
 - Increase expression of osteocalcin
 - Increase bone resorption
 - Increase bone formation
 - Rapid severe cancellous bone loss
 - Effects mediated by T lymphocytes
 - PTH may have permissive effect
 - Bone loss prevented by antiresorptive agents
-

^aThese observations are based primarily on animal studies.

Tacrolimus (FK506)

FK506 is a macrolide that binds to an immunophilin, FK binding protein, and blocks T-cell activation in a manner similar to cyclosporine. FK506 has been shown to cause bone loss in the rat model similar to that which occurs with cyclosporine (Table 2) (30). The bone loss is accompanied by similar biochemical and histomorphometric alterations. Therefore, the incidence of bone loss and fractures in organ transplant recipients managed with FK506 may not differ significantly from those managed with cyclosporine. FK506 is being used predominantly for liver transplantation. Liver transplantation, particularly in primary biliary cirrhosis patients managed with cyclosporine, produces a very high rate of fractures (31). It remains unclear whether FK506 will confer any benefit over cyclosporine with regard to fracture incidence. However, rapid bone loss occurs after cardiac (4) and liver transplantation (3), when tacrolimus is used for immunosuppression.

Azathioprine

Short-term administration of azathioprine does not cause bone loss in the rat model (32). However, there is an associated decline in serum osteocalcin values, which may represent an inhibitory effect on osteoblast function at a particular stage of development (32). The long-term consequences of azathioprine administration in this model are unknown. However, no adverse effects of azathioprine administration alone on bone mass have been reported in human subjects.

Sirolimus (Rapamycin)

Rapamycin is a macrocyclic lactone that is structurally similar to FK506 and binds to the same binding protein. Nevertheless, the mechanism by which rapamycin induces immunosuppression is distinct from both FK506 and cyclosporine. Studies in the rat model demonstrate that rapamycin has no adverse effect on bone (33). However, in large doses, rapamycin caused hypogonadism in male rats that resulted in severe osteopenia with fractures (33). This effect appears to be independent of alterations in the calmodulin-calcineurin phosphatase pathway. When combined with low-dose cyclosporine, rapamycin also has been shown to be bone sparing in rat studies (34). Thus combining immunosuppressive agents in lower doses may provide hope for achieving adequate immunosuppression while protecting the skeleton.

Mycophenolate Mofetil and Other Drugs

Mycophenolate displays no deleterious effect on bone in the rat model and should pose no problem when administered as an antirejection therapy (35). This drug is rapidly replacing azathioprine in many transplantation programs. There is little or no information available on the skeletal effects of other immunosuppressant agents such as mizoribine, deoxyspergualin, brequinar sodium, liflunomide, and azaspirane.

EFFECT OF TRANSPLANTATION ON BONE AND MINERAL METABOLISM

Causes of Bone Loss Before Transplantation

In many cases, individuals with chronic diseases severe enough to warrant organ transplantation have already sustained considerable bone loss (Table 3). The majority of candidates for organ transplantation have one or more accepted risk factors for osteoporosis. Such risk factors commonly include general debilitation, loss of mobility and physical inactivity, poor nutrition and cachexia. They are commonly exposed to drugs known to cause bone loss, such as heparin, loop diuretics, excessive doses of thyroid hormone and anticonvulsants. Some have received glucocorticoids before transplantation. Postmenopausal women and many of the premenopausal women are estrogen deficient. Similarly, men with chronic illness often have gonadal dysfunction, secondary to hypogonadotrophic hypogonadism. When the disease is present during childhood or adolescence, as is the case with cystic fibrosis or congenital heart disease, there may be interference with the attainment of peak bone mass during adolescence. It is essential to consider the possibility that bone mass may be reduced before transplantation. Consideration of particular issues related to transplantation of specific organs follows.

Kidney and Kidney-Pancreas Transplantation

SKELETAL STATUS BEFORE TRANSPLANTATION

Bone disease is almost universal in patients who undergo renal transplantation. Renal osteodystrophy is a general term that encompasses all the bone histological alterations that may occur in uremic patients (36). In a given individual, there may be evidence of hyperparathyroidism with or without osteitis fibrosa, osteomalacia, low turnover, or adynamic bone disease due to aluminum accumulation (or other as yet poorly understood factors), osteosclerosis particularly of the vertebrae, and β -macroglobulin amyloidosis. Many patients will have "mixed" renal osteodystrophy, a combination of one or more of the aforementioned lesions. Other factors that may affect the skeletal integrity of patients with ESRD include type I diabetes, hypogonadism secondary to uremia, and diseases such as systemic lupus erythematosus. Several drugs used routinely in the management of patients with renal disease, such as loop diuretics and aluminum containing phosphate binders, can also affect bone and mineral metabolism. In addition, some patients who are candidates for transplantation may have had previous exposure to glucocorticoids or cyclosporine as therapy for immune complex nephritis or other diseases and thus may already have sustained significant bone loss prior to transplantation.

Several cross-sectional studies have documented that osteoporosis and low bone mass are present in a significant proportion of patients on chronic dialysis (Table 3) (37–39). A history of fractures is also common in dialysis-dependent patients (40–42). Vertebral

Table 3
Osteoporosis Before and After Solid Organ and Bone Marrow Transplantation

Type of transplantation	Prevalence before transplantation		Prevalence after transplantation		Bone loss: first post-transplant year		Fracture incidence
	Osteoporosis ^a	Fracture	Osteoporosis ^a	Fracture			
Kidney ^b	8–49%	Vertebral: 3–21% Peripheral: 35%	10–40%	Vertebral: 3% Peripheral: 11–49%	Spine: 3–10% Hip: 5%		Vertebral: 3–10% Peripheral: 10–50%
Heart	8–10%	Vertebral: 18–50%	25–50%	Vertebral: 20–36%	Spine: 6–8% Hip: 8–12%		Vertebral: 10–35% Peripheral: 4%
Liver	8–43%	Vertebral: 20–25%		Vertebral: 29%	Spine: 2–24% Hip: 8%		Vertebral: 21–65%
Lung	30–35%	Vertebral: 14–49% Peripheral: 41%	57–67%	42%	Spine: 4–5% Hip: 4–5%		Vertebral and nonvertebral: 25–40%
Bone marrow	NA ^c	NA	4–18%	NA	Spine: 2–7% Hip: 7–12%		11%

^aAccepted definitions included BMD of spine and/or hip (by dual x-ray adsorptiometry) ≥ 2 SD below age- and sex-matched control or ≥ 2.5 SD below young normal controls, or BMD below fracture threshold.

^bDefinition of osteoporosis also included BMD of predominantly cortical sites such as the femoral shaft or proximal radius that are adversely affected by excessive PTH secretion.

^cNA, data not available.

fractures were present in 21% of Japanese hemodialysis patients (40). Approximately 34% of 68 hemodialysis had a history of previous fracture (41). The rate of fractures was reported to be 0.1 fractures per dialysis year in dialysis patients with osteitis fibrosis and 0.2 fractures per dialysis year in patients with adynamic bone disease (43). Risk factors for low bone mineral density (BMD) and fractures include female gender, Caucasian race, hyperparathyroidism, adynamic bone disease, prior renal transplantation, secondary amenorrhea, and type I diabetes.

PREVALENCE OF OSTEOPOROSIS IN KIDNEY TRANSPLANT RECIPIENTS

Low bone density measurements have been reported in several cross-sectional studies of patients who have undergone renal transplantation (Table 3). For example, vertebral bone mineral density was below the fracture threshold in 23% of 65 renal transplant recipients studied an average of 4 yr after transplantation (44). Female gender, postmenopausal status and cumulative prednisone dose were independent predictors of low bone density (44). Similarly, vertebral bone mineral density was more than two standard deviations below age- and sex-matched controls (Z score ≤ -2.0) in 41% of patients studied 6–195 mo after renal transplantation (45). The severity of osteopenia increased with time since transplantation and with increasing PTH concentrations. Pichette et al. found that lumbar spine and femoral neck bone density were more than two standard deviations below age- and sex-matched controls in 28.6% and 10.5% of 70 kidney transplant recipients studied an average of eight years after transplantation (46). Of their female subjects, osteopenia (by WHO criteria, T Score between -1.0 and -2.5) was present at the lumbar spine and femoral neck in 75% and 65%, respectively; osteoporosis (T score < -2.5) was present at the lumbar spine and femoral neck in 33 and 10%, respectively. Other studies have shown similar results (47–49).

BONE LOSS AFTER KIDNEY TRANSPLANTATION

Prospective longitudinal studies suggest that the majority of the bone loss observed in renal transplant recipients occurs during the early months to years after grafting (Table 3). In 1991, Julian et al. reported a decrease in lumbar spine BMD of $6.8 \pm 5.6\%$ at 6 mo and $8.8 \pm 7.0\%$ at 18 mo after transplantation (50). Moreover by 18 mo, bone density was below the “fracture threshold” in 10 of 17 patients. Bone biopsies performed prior to transplantation revealed typical changes of hyperparathyroidism. However, by 6 mo after transplantation, the histomorphometric picture was more typical of a glucocorticoid effect, demonstrating osteoblast dysfunction and decreased mineral apposition (50).

Since that initial report, several prospective studies have confirmed the bone loss reported by Julian and colleagues (51–55). The rate of bone loss is greatest during the first 6 mo after transplantation and at sites where cancellous bone predominates, such as the lumbar spine. The rate of lumbar spine bone loss varies between 3 and 10%. There appears to be a gender difference in the site at which bone is lost (52,53). Men have been shown to lose more bone at the proximal femur than women in the first few months after transplantation. In contrast, radial bone density increased in men at 6 mo post-transplantation but not in women.

The pathogenesis of bone loss after renal transplantation is complex. The majority of studies have found that glucocorticoid dose correlates positively with bone loss. There is also some evidence in the literature to support a role for cyclosporine in the pathogenesis of the high turnover state often apparent in renal transplant recipients by one year after renal transplantation (56–58).

FRACTURE AFTER KIDNEY TRANSPLANTATION

In addition to the declines in bone density, fractures also occur with increased frequency after renal transplantation (Table 3). Fractures affect appendicular sites (feet, ankles, long bones, hips) more commonly than axial sites (spine, ribs). A recent study determined that non-vertebral fractures are fivefold more common in males aged 25–64, and 18-fold and 34-fold more common in females aged 25–44 and 45–64, respectively, who have had a renal transplant than they are in the normal population (59). Fractures are particularly common in patients who receive kidney or kidney-pancreas transplants for diabetic nephropathy (60–62). In a retrospective study of 35 kidney-pancreas recipients, approximately half had sustained from one to three symptomatic, nonvertebral fractures by the end of the third posttransplant year (62). Vertebral fractures have been reported in 3–10% of nondiabetic patients after renal transplantation (46,49).

AVASCULAR NECROSIS

Avascular necrosis occurs commonly after renal transplantation (49,63–69). The incidence in children is 6% (63,66) and in adults is 8%. The hip is the most commonly affected site. While the association of avascular necrosis with glucocorticoids is well-established, cyclosporine has also been incriminated in producing avascular necrosis and bone pain of the hip and other weight bearing bones, such as the knees (70). The known vasospastic or vasoconstrictive properties of cyclosporine may contribute to the development of avascular necrosis. The cost of treating fractures and avascular necrosis (among other steroid-related side effects) has recently been reported in a cohort of 50 renal transplant recipients. The 10-yr cost of treating peripheral fractures was \$4300 and for osteonecrosis of the hip, \$61,700 (71,72); the higher cost of the latter likely reflects the use of magnetic resonance imaging for diagnosis and necessity for core decompression or total hip replacement.

MINERAL METABOLISM AND BONE TURNOVER AFTER KIDNEY TRANSPLANTATION

Predictable changes occur in biochemical indices of mineral metabolism and bone turnover after renal transplantation (50,73). PTH levels frequently remain elevated for some time after transplantation and may never completely normalize. Hypercalcemia and hypophosphatemia, related to persistent parathyroid hyperplasia and elevated PTH levels, occur commonly during the first few months. In most patients, these biochemical abnormalities are mild, well-tolerated, and resolve within 1 yr. Biochemical markers of bone formation are abnormal before and after renal transplantation (73). In general, formation markers are elevated, correlate directly with serum PTH and are not related to renal function, CsA or glucocorticoid doses. The biochemical evidence for increased bone turnover has been confirmed by histomorphometric data.

Cardiac Transplantation

SKELETAL STATUS BEFORE TRANSPLANTATION

Risk factors that may predispose patients with end stage cardiac failure to bone loss even before transplantation include exposure to tobacco, alcohol and loop diuretics, physical inactivity, hypogonadism and anorexia which may contribute to dietary calcium deficiency. Hepatic congestion and prerenal azotemia may also affect mineral metabolism. Although on average bone density of patients awaiting cardiac transplantation may not differ significantly from normal, it has been observed that approx 8–10% fulfill World Health Organization criteria for osteoporosis (Table 3; Fig. 1) and 40–50% have osteopenia or low bone mass (74–76).

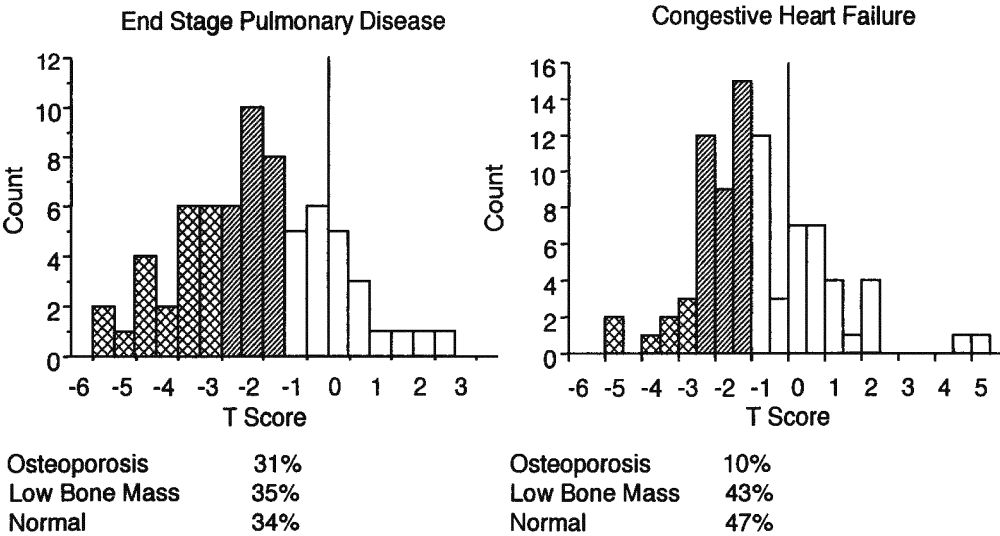


Fig. 1. Frequency distribution of bone mineral density (BMD) measurements of the lumbar spine in patients with end-stage pulmonary disease awaiting lung transplantation (A) and in patients with congestive heart failure (B). The data are expressed as T scores that relate BMD measurements of individual patients to those of a young normal population of the same gender. T-score measurements falling between -1 and -2.5 SD below the mean (hatched bars) indicate low bone mass or osteopenia while those below -2.5 SD below the mean (crosshatched bars) indicate osteoporosis.

PREVALENCE OF OSTEOPOROSIS IN HEART TRANSPLANT RECIPIENTS

Osteoporosis and fractures constitute a major cause of morbidity after cardiac transplantation. In cross-sectional studies, the prevalence rate of vertebral fractures in cardiac transplant recipients (Table 3) ranges between 18 and 50% and moderate to severe bone loss is present in a substantial proportion of subjects at both lumbar spine and the femoral neck (59,75–87).

BONE LOSS AFTER HEART TRANSPLANTATION

The pattern of bone loss after cardiac transplantation is similar to that observed after renal (50–55). Prospective longitudinal studies have documented rates of bone loss ranging from 2.5 to 20%, predominantly during the first three to 12 mo after transplantation (Table 3) (1,4,84,88–96). Despite the predilection for glucocorticoids to affect the cancellous bone of the vertebrae to a greater extent than other sites, we have reported that there is as much or more bone loss at the hip, a site with more cortical bone than the vertebral bodies. Moreover, while bone loss at the lumbar spine slows or stops after the first six months, femoral neck bone loss continues during the second half of the first year after transplantation (88). There are very few longitudinal data available on the pattern of bone loss during the second year after transplantation. However, our data suggests that the rate of bone loss slow or stops in the majority of patients, with some recovery at the lumbar spine noted during the third year of observation (88). Bone loss also slows at the hip after the first year; however, in contrast to the spine, there has been no significant recovery by the fourth post-transplant year.

FRACTURE AFTER HEART TRANSPLANTATION

Fragility fractures are strikingly common, particularly during the phase of rapid bone loss that characterizes the first post-transplant year (Table 3). In a prospective observa-

tional longitudinal study, 36% of patients (54% of the women and 29% of the men) suffered one or more fractures in the first year despite daily supplementation with calcium (1000 mg) and vitamin D (400 IU) supplementation (97). Although the majority of the fractures affected the vertebral bodies, two patients suffered multiple rib fractures and two had fractures of the femoral neck. The mean time to first fracture was 4 mo, with most patients sustaining their initial fracture during the first 6 mo. Lower pretransplant BMD and female gender were associated with a trend toward increased fracture risk. In men, however, it was the rate of bone loss after transplantation rather than the pretransplant bone density that was associated with fracture risk. It was also notable that many patients fractured who had normal pretransplant BMD and thus it was not possible to predict who would fracture either on the basis of pretransplant BMD or any other demographic or biochemical parameter (97). A European study of 159 cardiac transplant recipients reported similar fracture incidence with approx 30% of cardiac transplant recipients sustaining vertebral fractures during the first three years (98). These studies underscore the need for a complete bone evaluation and bone mass measurements prior to, or immediately after transplantation, as well as aggressive intervention to prevent bone loss and fractures in all patients regardless of age, sex, or pretransplant bone density.

MINERAL METABOLISM AND BONE TURNOVER AFTER HEART TRANSPLANTATION

Biochemical changes after cardiac transplantation include sustained increases in serum creatinine (88,90,99) and decreases in 1,25 dihydroxyvitamin D concentrations (88). On average, serum testosterone concentrations decrease in men with recovery by the sixth post-transplant month (88,90,99). Serum osteocalcin falls precipitously and there is a sharp increase in markers of bone resorption (hydroxyproline and pyridinium crosslink excretion) during the first three months with return to baseline levels by the sixth month (88,99). This biochemical pattern coincides with the period of most rapid bone loss and highest fracture incidence and suggests that the early post-transplant period is associated with uncoupling of formation from resorption. In contrast, several studies of subjects treated with high doses of glucocorticoids alone confirm the decrease in serum osteocalcin but found no increase in markers of bone resorption (100,101). This suggests that the pathogenesis of early bone loss after cardiac transplantation may be related both to the well-known inhibitory effects of glucocorticoids on bone formation, and to a effect of cyclosporine A or some other agent to increase bone resorption. There is also evidence for a high bone turnover state later in the post-transplant course perhaps due to cyclosporine, characterized by elevations in both serum osteocalcin and urinary excretion of resorption markers (75,77,82,86,87,90,92,99). In a recent cross-sectional study, Eastell and colleagues (82) evaluated 50 men, ranging from 0.5 to 47 mo after cardiac transplantation. They concluded that bone turnover is increased after cardiac transplantation, and that the increased bone turnover is due in part to secondary hyperparathyroidism related to renal impairment. Thus biochemical changes later in the post-transplant course may be mediated, at least in part, by cyclosporine A-induced renal insufficiency, although other etiologies cannot be excluded.

Liver Transplantation

SKELETAL STATUS BEFORE TRANSPLANTATION

Patients with liver failure have multiple risk factors that may predispose to low bone mineral density before transplantation and fracture after transplantation (102). Many

patients with end-stage liver disease who are listed for liver transplantation have prevalent osteoporosis (Table 3), as evidenced by low bone mineral density (BMD) and fragility fractures (*103,104*). Osteoporosis and abnormal mineral metabolism have been described in association with alcoholic liver disease (*105,106*), hemochromatosis (*107*), steroid-treated autoimmune chronic active hepatitis (*108,109*), postnecrotic cirrhosis (*110*) and particularly in chronic cholestatic liver diseases such as biliary cirrhosis (*31,111*). Thus, candidates for liver transplantation frequently have significant pre-transplant skeletal demineralization and fractures that place them at increased risk for fracture both before and after surgery. A study of 58 patients with cirrhotic end-stage liver disease referred for liver transplantation (*103*), reported that 43% had osteoporosis (defined as Z score > 2 S.D. below age-matched controls or presence of vertebral fractures). Serum 25-OHD, 1,25(OH)₂D, intact PTH and osteocalcin (a marker of bone formation) were lower and urinary hydroxyproline excretion (a marker of bone resorption) was higher in cirrhotic patients than controls. Male patients had lower serum testosterone levels than controls. Another study of 56 liver transplant recipients revealed that 23% had osteoporosis that antedated transplantation (*112*).

Serum osteocalcin concentrations are generally low in primary biliary cirrhosis and other forms of chronic liver disease and histomorphometric data have confirmed that bone formation is decreased in patients with primary biliary cirrhosis (*113–115*). Crosbie et al. have noted biochemical evidence of both decreased bone formation and increased bone resorption in patients with chronic liver disease (*104*). While serum osteocalcin appears to be a valid marker of bone formation in cholestatic liver disease, the utility of collagen-related markers of bone turnover has recently been called into question (*115*). In fibrotic liver diseases, the synthesis of type I collagen is markedly increased. Guanabens et al. recently evaluated osteocalcin and collagen-related markers of bone formation (carboxy-terminal and amino-terminal propeptides of type I collagen) and resorption (serum tartrate-resistant acid phosphatase or TRAP, cross-linked carboxy-terminal telopeptide of type I collagen, and urinary excretion of pyridinium crosslinks, hydroxyproline, N- and C-telopeptides) in 34 women with primary biliary cirrhosis (*115*). Of these, serum osteocalcin was significantly lower than controls and tartrate-resistant acid phosphatase (TRAP) was similar to controls. In contrast, all of the collagen-related markers of bone turnover were significantly higher in patients than controls and all correlated with indices of liver fibrogenesis and disease severity rather than with osteocalcin and TRAP. Thus, collagen-related bone turnover markers appear influenced by liver, rather than bone, collagen metabolism and do not reflect skeletal turnover in patients with liver disease.

BONE LOSS AND FRACTURE AFTER LIVER TRANSPLANTATION

Rates of bone loss and fracture vary considerably after liver transplantation (Table 3), but are often extremely high (*31,98,112,116–124*). In 1988, Haagsma et al. (*116*) reported 26 adults who were evaluated with skeletal radiographs prior to and after liver transplantation. All patients were maintained on 1- α cholecalciferol (1 mg/d) and oral calcium supplements. Atraumatic vertebral fractures developed in 38%, mainly during the second three months after transplantation. In 1992, Arnold et al. (*119*) evaluated 48 patients before and after liver transplantation with bone densitometry and spinal radiographs. Lumbar spine BMD declined significantly during the initial 3–6 mo, after which bone

mass stabilized in the majority of patients. Atraumatic fractures developed in 31% and the mean time to fracture was 6 mo. Mean pretransplant lumbar spine and forearm bone mass tended to be lower in the fracture patients than in those who did not fracture. Similarly, McDonald et al. (118) reported that spine BMD decreased by 24% during the first 3 mo after liver transplantation, with no significant change during the remainder of the first year. Six of 17 patients (35%) sustained atraumatic fractures during the first 6 mo. These investigators also noted that pretransplant lumbar spine bone mass did not differ between patients who later fractured and those who did not. However, BMD at 3 mo was lower in the fracture group. In 1994, Navasa et al. (123) reported that symptomatic spine fractures developed in 24% of 91 patients during the first year (median 6 ± 4 mo) after liver transplantation. In that study, fracture risk was found to be higher in patients with previous atraumatic fractures, primary biliary cirrhosis, or a previous liver transplant. Women with primary biliary cirrhosis, a disease characteristically associated with low turnover osteoporosis, may be at even greater risk to sustain fractures after liver transplantation. Eastell et al. (31) reported that 13 of 20 of women (65%) with this disorder suffered fractures during the initial year after transplantation. Median lumbar spine BMD declined at a rate of 18%/yr during the first 3 mo after transplantation and then increased slightly between 3 and 12 mo. Similar observations were made by Porayko et al. in patients with primary biliary cirrhosis (117). Meys et al., in a recent cross-sectional study, reported that the prevalence rate of vertebral fracture in 31 liver transplant recipients studied 1 yr after transplantation was significantly higher (29 vs 8%) than in 33 patients studied prior to liver transplantation (122). These authors followed alterations in bone mass in 16 patients and observed a mean decrease of 3.5% in the lumbar spine BMD one year after grafting. No information was given on the incidence rate of fracture. More recently, Keogh et al. reported that femoral neck BMD fell by 8% and lumbar spine BMD by 2% after liver transplantation (125).

In summary, lumbar spine BMD falls by 2–24%, primarily in the initial year after liver transplantation. However, at least in patients with primary biliary cirrhosis, bone loss appears to stop after 3 mo with gradual improvement by the second and third post-transplant years. Fracture incidence is also highest in the first year and ranges from 24–65%, the latter in a group of women with primary biliary cirrhosis. The vertebrae and ribs are the most common fracture sites. No pretransplant indicator reliably predicts fracture risk in the individual patient. However, retransplanted patients, those with primary biliary cirrhosis and those with previous fragility fractures are at increased risk. Eastell et al. reported that despite the high incidence of fractures in liver transplant recipients, bone mass recovers and bone histology normalizes with increasing survival time after transplantation (31), and other investigators have shown that there is improvement in BMD in long-term liver transplant recipients (126). This, however, has not been a uniform finding and other studies have found continued losses rather than recovery (121,127). Depending upon the bone density at the time of transplantation, these patients may always be at risk for fractures as survival rates and duration increase.

MINERAL METABOLISM AND BONE TURNOVER AFTER LIVER TRANSPLANTATION

Studies of calciotropic hormone levels and bone turnover markers after liver transplantation are limited. Compston et al. reported a significant rise in serum intact PTH during the first 3 mo after liver transplantation, although levels did not exceed the upper

limit of the normal range (128). Others have found intact PTH levels to be within the normal range in liver transplant recipients (121,122,129). With respect to bone turnover, markers of bone formation (osteocalcin and carboxyterminal peptide of type I collagen) were found to be significantly higher in 120 liver transplant recipients than in a normal control population (129). Similarly, serum osteocalcin was higher than age- and sex-matched controls in 18 men and nine postmenopausal women after liver transplantation and urinary hydroxproline excretion was also elevated (122). Significant increases in serum osteocalcin were not observed in one study in which levels were obtained before and 1 mo after transplantation (130). However, in other studies of longer duration, serum osteocalcin levels were found to increase substantially during the first post-transplant year (124,131). The balance of the data thus suggests that low bone turnover observed in many patients with liver failure converts to a high turnover state that persists indefinitely after liver transplantation.

As is the case with renal and cardiac transplantation, the independent role of glucocorticoids and calcineurin phosphatase inhibitors in the pathogenesis of bone disease in liver transplant patients is difficult to assess since single drug therapy is uncommon. The mechanism of bone loss after liver transplantation has been studied by bone biopsy in 21 patients, evaluated with tetracycline labeling and transiliac crest bone biopsy, before and 3 mo after transplantation. Before transplantation, a low turnover state was observed, with decreased wall width and erosion depth. Postoperative biopsies showed high turnover with increased formation rates and activation frequency, and a trend toward increased indices of resorption (132). In an earlier study, these investigators documented a significant increase in parathyroid hormone concentrations after liver transplantation (128). While increased PTH could account for these histomorphometric findings, similar effects are observed in animals treated with calcineurin inhibitors without a rise in PTH concentrations.

Lung Transplantation

SKELETAL STATUS BEFORE LUNG TRANSPLANTATION

Hypoxemia, tobacco use, and prior glucocorticoid therapy are frequent attributes of candidates for lung transplantation and may contribute to the pre-transplant bone loss (Table 3; Fig. 1) which is particularly common in these patients (133,134). Cystic fibrosis (CF), a common reason for lung transplantation, is itself associated with osteoporosis and fractures due to pancreatic insufficiency, vitamin D deficiency and calcium malabsorption and hypogonadism (135). A greatly increased rate of all fractures and severe kyphosis has been reported in adults with cystic fibrosis (136). We have observed that vitamin D deficiency is extremely common in CF patients, despite supplementation; bone density was significantly lower in the D-deficient patients (137). Two cross-sectional studies have found that low bone mass and osteoporosis are present in 45–75% of candidates for lung transplantation (133,134). In both studies, glucocorticoid exposure was inversely related to BMD. Vertebral fracture prevalence was 29% in patients with emphysema and 25% in patients with CF (134).

BONE LOSS AND FRACTURE AFTER LUNG TRANSPLANTATION

Few studies have prospectively evaluated patients after lung transplantation (Table 3). A recent study of 12 patients demonstrated an average 4% decrease in lumbar spine BMD during the first 6 mo despite calcium and 400 IU of vitamin D (138). Two men sustained multiple vertebral fractures. Another study confirmed that osteopenia was

common before lung transplantation (139). Despite calcium and vitamin D, there was a further decrease of approx 5% in both lumbar spine and femoral neck BMD during the first 6–12 mo after lung transplantation and fractures developed in 18% of 28 patients (139). In a retrospective analysis of 33 lung transplant recipients, who had survived at least 1 yr after grafting, BMD was markedly decreased and 42% had vertebral fractures (140). Moreover, our experience suggests that as many as 37% of lung transplant recipients suffer fragility fractures and significant bone loss during the first post-transplant year despite receiving antiresorptive therapy (141). Risk factors for fracture and bone loss included female gender, low pretransplant lumbar spine BMD, pretransplant glucocorticoid therapy, and higher bone turnover after transplantation.

Bone Marrow Transplantation

Bone marrow transplantation is performed with increasing frequency and for expanding indications. In preparation for transplantation, patients receive myeloablative therapy (alkylating agents and/or total body irradiation) and commonly develop profound and frequently permanent hypogonadism, which could certainly cause bone loss. After transplantation, patients may receive glucocorticoids, methotrexate, or cyclosporine A, alone or in combination. Low BMD was first reported after bone marrow transplantation by Kelly et al. (142). Since then several cross-sectional studies have confirmed low total body BMD (143) or bone mineral content (BMC) (144) (by DXA) and lumbar spine BMD (by computed tomography) (145) in bone marrow transplant recipients (Table 3). However, only those who were less than 18 yr old at the time of transplantation were affected (143). Two studies have documented that bone mass is low in hypogonadal women after bone marrow transplantation (146,147) and that hormone replacement therapy is associated with significant increases in bone mineral density (146).

With respect to timing of bone loss after bone marrow transplantation, a study of 9 adults undergoing 6 mo of high dose glucocorticoid and CSA therapy for graft-vs-host disease (GVHD) observed significant lumbar spine bone loss in most patients (148). Ebeling et al. found that low bone mass antedates bone marrow transplantation, particularly in subjects with prior glucocorticoid exposure and that postransplant bone loss is particularly severe in patients who undergo allogeneic bone marrow transplantation, probably because of their increased propensity for GVHD (149). Valimaki et al. followed a group of patients who had undergone allogeneic bone marrow transplantation for 6 mo ($n = 44$) and 12 mo ($n = 36$) after grafting. Although some received calcium and vitamin D and some received calcitonin, there was no discernable difference in rates of bone loss; therefore the groups were combined. BMD decreased by approx 6% at the lumbar spine and 7% at the femoral neck (150). Kang et al. found that lumbar spine BMD decreased by 2.2% and femoral neck BMD by 6.2% during the first year (151). Similarly, Kashyap et al. found that lumbar spine BMD decreased by 3.0% and femoral neck BMD by 11.6% during the first 12–14 mo (152). There appears to be little bone loss after the first year (145).

In the study by Valimaki et al., bone turnover markers were notable for decreased formation and increased resorption during the first 3 mo (90); this is a pattern consistent with uncoupling of formation from resorption, and is similar to that observed after cardiac transplantation (88). After 3 mo, there was recovery of bone formation markers and generally elevated turnover during the latter half of the year (150). Similar elevations of bone turnover markers have also been observed by other investigators both before and after bone marrow transplantation (145,153,154), as after solid organ transplantation (73).

Table 4
Evaluation of the Candidate for Organ Transplantation

In all candidates:

- Assess risk factors for osteoporosis
- Measure bone densitometry (BMD) of spine and hip by dual energy x-ray adsorptiometry
- Obtain thoracic and lumbar spine radiographs

If BMD testing reveals osteoporosis or there are prevalent vertebral fractures:

- Serum calcium, parathyroid hormone, 25-hydroxyvitamin D, thyroid function tests (see text)
- In men, serum total and/or free testosterone, FSH, and LH
- Urine for calcium and markers of bone resorption (optional)

EVALUATION AND MANAGEMENT OF CANDIDATES FOR TRANSPLANTATION

Evaluation

There are now abundant data documenting the high prevalence of bone disease in candidates for all types of transplantation. Therefore the possibility of significant bone disease should be addressed before transplantation so that potentially treatable abnormalities of bone and mineral metabolism may be addressed and the skeletal condition of the patient optimized before transplantation (Table 4). Risk factors for osteoporosis should be assessed. These include a family history of osteoporosis, medical conditions (thyrotoxicosis, renal disease, rheumatological, and intestinal disorders), poor lifestyle choices (physical inactivity, dietary calcium and vitamin D deficiency, excessive caffeine and alcohol intake, tobacco use) and exposure to certain drugs (diphenylhydantoin, lithium, loop diuretics, glucocorticoids, prolonged and large doses of heparin, thyroid hormone). Additional risk factors important in women include premature menopause, postmenopausal status, history of anorexia nervosa or prolonged episodes of amenorrhea. In men, it is important to exclude hypogonadism. A physical examination should focus upon diseases that predispose to osteoporosis such as hypogonadism, thyrotoxicosis and Cushing's syndrome. Risk factors for falling (sight, hearing, balance and muscle strength, psychotropic drugs) should also be assessed.

Bone density of the spine and hip is the most important test to obtain prior to transplantation. Radiographs of the thoracic and lumbar spine are also important since risk of future fracture is greater in patients with prevalent vertebral fractures. A battery of biochemical tests is unnecessary if the bone density measurement is normal and supplementation with calcium and vitamin D is planned. However, if the pretransplant bone density is low, a thorough biochemical evaluation can alert the physician to the etiology of low bone mass and guide appropriate therapy. In such instances, the biochemical evaluation should include a chemistry panel (serum electrolytes, creatinine, calcium, phosphorus, alkaline phosphatase), thyroid function tests, intact PTH and vitamin D metabolites. In men, total and free testosterone, FSH, and LH concentrations should be obtained. Markers of bone formation (serum osteocalcin and bone specific alkaline phosphatase) and resorption (urinary deoxypyridinoline, C- or N-telopeptide excretion) can also be measured (Table 4) to assess bone turnover status.

Although pretransplant BMD does not reliably predict fracture in individual patients, low pretransplant BMD probably increases fracture risk, particularly in postmenopausal

Table 5
Short-Term Prevention of Osteoporosis in Transplant Recipients

-
- Measure BMD before or immediately after transplantation, and every 6 mo for 2 yr
 - Refer for evaluation/therapy all patients with low bone mass (T score between -1.0 and -2.5) or osteoporosis (T score < -2.5)
 - Endeavor to use the lowest dose of glucocorticoids possible
 - Consider alternate therapies for rejection (e.g., OKT3)
 - Ensure calcium intake of 1500 mg/d both before and after transplantation
 - Ensure a vitamin D intake of 400–1000 IU, or as needed to maintain serum 25-OHD concentrations in the upper half of the normal range
 - Prescribe a physical rehabilitation program both before and after transplantation
 - Replace gonadal steroids in hypogonadal women and men
 - Begin antiresorptive therapy, preferably a bisphosphonate, before transplantation in patients with antecedent osteoporosis or low bone mass
 - Begin antiresorptive therapy, preferably a bisphosphonate, immediately after transplantation in patients with normal or low bone mass and continue for at least the first year
-

women. Although no data are yet available that therapy before transplantation improves BMD and reduces post-transplant fracture risk, antiresorptive therapy clearly increases BMD and reduces fracture rates in other patients. Therefore, individuals awaiting transplantation who meet World Health Organization criteria for diagnosis of osteoporosis (T Score < -2.5), osteopenia or low bone mass (T score between -1.0 and -2.5) should be evaluated and treated similarly to others with, or at risk, for osteoporosis (Table 5).

During the waiting period before transplantation, rehabilitation therapy should be prescribed as tolerated to maximize conditioning and physical fitness. All transplant candidates should receive the Recommended Daily Allowance of vitamin D (400–800 IU) and elemental calcium (1000–1500 mg, depending on dietary intake and menopausal status). Either calcium citrate or calcium carbonate is acceptable; however, the carbonate form should be taken with food to enhance absorption and may cause constipation. Hormone replacement therapy should be considered in all postmenopausal women, as well as in premenopausal amenorrheic women provided there are no contraindications to such therapy. Hypogonadal men should also be offered testosterone replacement. Generally accepted guidelines for gonadal hormone replacement should apply to these patients.

Patients who are found to have osteoporosis before transplantation may begin antiresorptive therapy with a bisphosphonate or a selective estrogen receptor modulator (SERM). The pretransplant waiting period is often long enough (1–2 yr) for there to be significant improvement in bone mass before transplantation. Patients with renal osteodystrophy should be managed in accordance with accepted clinical guidelines.

After transplantation, serum and urine indices of mineral metabolism are less crucial. However, monitoring may be useful to detect developing conditions that may contribute to bone loss (vitamin D deficiency or renal insufficiency with secondary hyperparathyroidism. Serum (and urinary) calcium must be monitored frequently if pharmacologic doses of vitamin D are used, in order to detect hypercalciuria or hypercalcemia. Measurement of bone density, on the other hand, remains important and should be performed at 6-mo intervals for the first 2 yr and annually thereafter. Bone biopsy may be necessary after renal transplantation since many experts remain reluctant to use bisphosphonates in patients with adynamic bone disease, despite lack of any data either to support or

Table 6
Management of the Long-Term Organ Transplant Recipient

In all patients:

- Assess risk factors for osteoporosis
- Measure bone densitometry (BMD) of spine and hip by dual energy x-ray absorptiometry
- Obtain thoracic and lumbar spine radiographs
- Begin calcium (500 mg TID) and vitamin D (400–800 ID QD) supplementation
- Recommend physical rehabilitation program

If BMD testing reveals osteoporosis or there are prevalent vertebral fractures:

- Serum calcium, parathyroid hormone, 25-hydroxyvitamin D, thyroid function tests
 - In men, serum total and/or free testosterone, FSH, and LH
 - Urinary/serum markers of bone resorption (optional)
 - Replace gonadal steroids in hypogonadal women and men, if appropriate
 - Begin antiresorptive therapy, preferably a bisphosphonate
-

exclude the use of these drugs in this setting. Although transiliac crest bone biopsy remains a research tool, more histomorphometric studies would be very helpful in confirming theories of the pathogenesis of transplantation osteoporosis.

PREVENTION OF TRANSPLANTATION OSTEOPOROSIS

The major principles that should guide therapy of transplantation osteoporosis are as follows. Rates of bone loss are most rapid immediately after transplantation. Fractures also occur very early after transplantation, sometimes within only a few weeks of grafting. Fragility fractures develop both in patients with low and those with normal pretransplant BMD. These principles have been demonstrated unequivocally after kidney, liver, heart, lung, and bone marrow transplantation. Moreover, prevention of the rapid bone loss that accompanies transplantation is likely to be considerably more effective in reducing the morbidity from fractures than waiting for fractures to occur before initiating therapy. Therefore, preventive strategies should be instituted immediately after transplantation both in patients with normal pretransplant BMD and those with low BMD who have not been treated previously (Table 5). In addition, the long-term transplant recipient with established osteoporosis and/or fractures should not be neglected (Table 6). Prospective controlled studies of therapies for prevention and treatment of transplantation osteoporosis are gradually beginning to appear in the literature, although the quality of published studies varies. The recommendations provided herein are also based upon experience with glucocorticoid-induced osteoporosis.

Available therapies of transplantation osteoporosis include antiresorptive drugs (bisphosphonates, calcitonin, and estrogen), those that stimulate bone formation (fluoride), as well as analogues of vitamin D and testosterone (Table 7). Since resorption markers increase after transplantation and correlate directly with rates of bone loss (73), attempts to prevent post-transplantation bone loss, and hopefully fractures, by inhibition of bone resorption are a logical approach.

Bisphosphonates

Bisphosphonates act by inhibiting osteoclastic bone resorption. This class of drugs is most commonly used to treat post-menopausal osteoporosis. However, they have also

Table 7
Specific Therapies
for Transplantation Osteoporosis

Bisphosphonates
Etidronate
Clodronate
Pamidronate
Alendronate
Risendronate
Vitamin D
Parent vitamin D (high dose)
25-OHD (Calcidiol)
1,25(OH) ₂ D (Calcitriol)
Hormone replacement therapy
Estrogen
Testosterone
Calcitonin
Fluoride

been used successfully both to prevent and to treat glucocorticoid-induced bone loss (155). Published studies include first, second, and third generation bisphosphonates such as etidronate (156), pamidronate (157), and, most recently, alendronate (158), and risedronate (159). Both alendronate and risedronate have been approved by the FDA for prevention and treatment of glucocorticoid-induced osteoporosis. Since transplantation osteoporosis can be considered one form of glucocorticoid-induced osteoporosis and since cyclosporine and tacrolimus-induced bone loss are characterized experimentally by increases in both formation and resorption, bisphosphonates offer considerable hope for prevention of transplantation osteoporosis.

Some (129,160–164) studies suggest that bisphosphonates can prevent bone loss and fractures after transplantation. In an open-label, pilot study of heart transplant recipients, a single intravenous dose of pamidronate (60 mg) was given during the first 2 wk after transplantation and followed by etidronate (400 mg, given cyclically for the first 14 d of every third month) for the remainder of the first year. Lumbar spine and femoral neck bone loss was prevented and fracture incidence significantly reduced compared to patients who received only calcium and 400 IU of vitamin D (162). In a small, open but randomized clinical trial, intravenous pamidronate was administered to kidney transplant recipients at time of grafting and again 1 mo later (160). Both lumbar spine and femoral neck bone loss were completely prevented in the treated group. In contrast, lumbar spine BMD fell by 6.4% and femoral neck BMD by 9% in the control subjects. Aris et al., in a randomized, controlled nonblinded trial, demonstrated that intravenous pamidronate (30 mg every 3 mo for 2 yr) was associated with 8% increases in spine and hip BMD in patients who underwent lung transplantation for cystic fibrosis (165). Unfortunately, however, fracture rates were very high (nine patients had long bone and four patients had vertebral fractures) and did not differ between the two treatment groups (165). Data from a retrospective study by Reeves et al. suggests that treatment with intravenous pamidronate before and every 3 mo after liver transplantation prevented symptomatic vertebral fractures in liver transplant recipients who had osteoporosis before transplantation (161).

Two studies suggested that bisphosphonates may not be efficacious (166,167). However, in both the only drug used was etidronate, the weakest available bisphosphonate. Clinical trials are currently underway with alendronate in prevention of osteoporosis after cardiac, liver, and renal transplantation and our own clinical experience suggests that this drug is effective after cardiac transplantation (163). The recent demonstration, in women with postmenopausal osteoporosis, that once weekly dosing with oral alendronate (70 mg) causes similar increases in BMD as daily dosing with 10 mg is of interest (168). Serious gastrointestinal side effects were less common in the weekly dosing group. Such regimens may prove very useful in transplant patients who have many gastrointestinal symptoms and take large numbers of medications. For such patients, the requirement to take oral bisphosphonates first thing in the morning and wait 30–60 min before eating or taking other medications is particularly inconvenient.

At present, bisphosphonates constitute the most promising approach to the prevention of transplantation osteoporosis. As with other forms of therapy, many issues remain to be resolved. These include the optimal drug and route of administration, whether continuous or intermittent (cyclical) therapy should be used, at what level of renal impairment these drugs should be avoided, whether they are safe in renal transplant recipients with adynamic bone disease and whether they are beneficial in the setting of pediatric transplantation.

Vitamin D and Analogs

Administration of vitamin D or its analogs is often recommended after transplantation (169). The exact mechanism by which vitamin D and its analogues may influence post-transplantation bone loss is uncertain. They may overcome glucocorticoid-induced decreases in intestinal calcium absorption, reduce the potential for secondary hyperparathyroidism, promote differentiation of osteoblast precursors into mature cells, or influence the immune system and potentiate the immunosuppressive action of cyclosporine (170,171). The last of these potential mechanisms is of particular interest since cyclosporine increases both production and serum levels of $1,25(\text{OH})_2\text{D}$ in the rat and mouse (172). However, in humans, no evidence has been found yet for a similar effect and in fact, serum concentrations of $1,25(\text{OH})_2\text{D}$ have been shown to fall after cardiac transplantation (88).

Since most of the observational studies of bone loss after organ transplantation have included at least 400 IU of parent vitamin D in the post-transplant regimen, it is clear that the RDA for vitamin D is not sufficient to prevent transplantation osteoporosis. Sambrook et al. (173) found calcitriol, the active metabolite of vitamin D, to be efficacious in patients with glucocorticoid-induced osteoporosis. These investigators have recently reported that calcitriol (0.5–0.75 mg/d) prevented spine and hip bone loss during the first 6 mo after heart or lung transplantation and was as effective as cyclic etidronate (174). However, Stempfle et al. found that the addition of a small dose of calcitriol (0.25 $\mu\text{g}/\text{d}$) to calcium supplementation and gonadal steroid replacement offered no benefit with regard to bone loss or fracture prevention after cardiac transplantation (80). Whether vitamin D should be prescribed as the parent compound in doses of 50,000 IU once a week or calcitriol 20 mg daily (83), or calcitriol 0.25 to 0.75 μg daily, or even at all is controversial.

Hypercalcemia and hypercalciuria are the major side effects of therapy of these agents. Either may develop suddenly and at any time during the course of treatment. Thus, frequent urinary and serum monitoring may be required. If hypercalcemia occurs, it must be recognized and reversed promptly because of the adverse effects on renal function and

the life threatening potential of a severely elevated serum calcium concentration. Supplemental calcium and any vitamin D preparations must be discontinued until the calcium normalizes. Although one may be tempted to permanently discontinue pharmacologic doses of vitamin D or its metabolites in view of the necessary serial monitoring and potential dangers, general opinion would be to recommence therapy at a lower dose.

In summary, given the requirement for serial monitoring and the narrow therapeutic window with respect to hypercalcemia and hypercalciuria, we regard pharmacologic doses of vitamin D and its analogues as adjunctive rather than primary therapy for the prevention and treatment of transplantation osteoporosis.

Calcitonin

Calcitonin has long been used to treat Paget's disease of bone, a disease characterized by focal areas of high bone turnover. In the therapy of osteoporosis, calcitonin has been shown to increase bone density in patients with high turnover osteoporosis. Moreover, this drug may have an analgesic effect upon acutely painful fractures and chronic pain due to multiple vertebral fractures. Despite many years of experience, the optimum dose, route of administration, and efficacy of continuous versus intermittent dosing remain unclear. Both injectable and inhaled calcitonin has been used successfully to treat glucocorticoid-induced bone loss in humans (175). Experimental work in the rat model has demonstrated that cyclosporine induced bone loss can be prevented by calcitonin (25) and it is reasonable to use calcitonin to protect against transplantation osteoporosis. However, literature on the use of calcitonin in preventing bone loss and fractures after transplantation is not consistent. The usual practice is to prescribe synthetic salmon calcitonin, 100 U daily by subcutaneous injection or intranasal calcitonin (100–200 IU) as soon as immunosuppressive therapy is begun. Grotz et al. randomly assigned long-term kidney transplant recipients with low bone density to receive intranasal calcitonin (200 IU for 2 wk every third month), clodronate (a bisphosphonate available in Europe) or no therapy (176). BMD increased similarly in all three groups. Valero et al. administered either injectable calcitonin or etidronate to long-term liver transplant recipients and found that lumbar spine bone density increased by 6–8% with no difference in efficacy between the drugs (129). Other investigators found nasal calcitonin to be ineffective after cardiac transplantation (164). Moreover, our clinical experience with injectable calcitonin suggests that it is relatively ineffective in preventing bone loss or fractures during the first year after transplantation.

Estrogen

Although estrogen is of theoretical benefit, no studies have been published regarding the effects of hormone replacement therapy in solid organ transplant recipients. Our own experience suggests that estrogen therapy alone, is not sufficient to prevent bone loss during the first post-transplant year in postmenopausal women. In postmenopausal women or premenopausal women with amenorrhea or irregular menses, estrogen replacement should be recommended provided that there are no contraindications. The dose is the same as that used for prevention or therapy of postmenopausal osteoporosis and may be given either orally or transdermally. In women with an intact uterus, progesterone must be prescribed in addition to prevent endometrial cancer. Continuous rather than cyclical therapy is preferred after transplantation, as estrogen enhances hepatic metabolism of cyclosporine (and presumably FK506) and theoretically may compro-

mise immunosuppression. Whether this occurs in patients is not known. Premenopausal amenorrheic women often begin menstruating after transplantation and estrogen therapy may often be discontinued 3–6 mo after surgery. For patients who cannot take estrogens, newer selective estrogen receptor modulators (SERMs) such as raloxifene (177) may be a suitable alternative.

Both estrogen (23) and raloxifene (24) have been shown to reduce cyclosporine-induced bone loss in the rat model. Few trials have evaluated the efficacy of estrogen in preventing bone loss and fractures after transplantation. However, a wealth of clinical data supports the protective effect of estrogens on the skeleton and one cross-sectional study suggests that estrogen and progesterone therapy are associated with higher bone density in women on glucocorticoids (178). In bone marrow transplantation, in which hypogonadism is a predominant feature, 12 mo of hormone replacement therapy (HRT) was associated with significantly increased bone density in a small number of women, without adversely affecting liver enzymes (146). There may also be an advantage to combining HRT with a bisphosphonate, particularly in light of a recent study (179) in which the addition of alendronate in a group of postmenopausal women on stable doses of HRT caused a significant increase in BMD.

Testosterone

Hypogonadism is common in men with chronic illness. Moreover, the suppressive effects of cyclosporine A and glucocorticoids on the hypothalamic-pituitary-gonadal axis often lower serum testosterone levels. Although testosterone usually normalizes by 6–12 mo after transplantation (88,99), approx 25% of men evaluated 1–2 yr after transplantation will have biochemical evidence of hypogonadism. Hypogonadism is known to cause osteoporosis in men. Moreover, men with low serum testosterone concentrations have been shown to lose bone more rapidly after cardiac transplantation (88,99).

In general, men who are truly hypogonadal should be treated with testosterone. However, there are no data on the effect of testosterone replacement on rates of bone loss after organ transplantation. It is also not known whether testosterone replacement is advisable to counter the generally transient decreases observed during the early months after transplantation. Potential benefits of testosterone therapy include increased lean body mass and hemoglobin, and improved BMD. Potential risks include prostatic hypertrophy, abnormal liver enzymes, and acceleration of hyperlipidemia in patients already prone to atherosclerosis from hypertension, diabetes, glucocorticoid and CsA therapy. Therefore it is necessary to monitor serum lipids and liver enzymes, and perform regular prostate examinations in men receiving testosterone.

Fluoride

Fluoride, one of the few drugs that can stimulate bone formation, is an appealing approach to disorders such as transplantation or glucocorticoid-induced osteoporosis, in which osteoblast inhibition may play a pathogenetic role. Meys et al. (83) used disodium monofluorophosphate (26.4 mg elemental fluoride) together with 1 g of elemental calcium and 25 mg (1000 IU) of calcidiol in cardiac transplant patients and compared these patients to another group who received the same dose of calcium and calcidiol without the fluoride compound. After 12 and 24 mo of therapy, there was no decline in lumbar spine bone mass in those treated with calcium and calcidiol. In contrast, the group that received fluoride demonstrated an increase in lumbar bone density of 12.5% after 12 and 29.5% after 24 mo,

respectively. Side effects were observed in 10 of 57 patients (gastric intolerance in five patients and lower limb pain in five patients). No hip, long bone, or vertebral fractures were seen in the second year of treatment in either group. However, increases in BMD in response to fluoride therapy are not consistently associated with improvement in bone quality and strength, or reduction in fracture rates. This study, although interesting, was open and uncontrolled, and no bone biopsy data were reported. Thus, the quality of the new bone was unknown. Moreover, high doses (50–75 mg/d) of sodium fluoride have troubling gastrointestinal side effects. The potential approval of slow-release sodium fluoride in the United States may provide alternative or adjunctive therapy for transplantation osteoporosis. While encouraging, controlled trials are still necessary to assess the true value of this particular fluoride preparation in transplantation osteoporosis.

New Therapeutic Options

Discussion of new advances in immune therapy that will prevent organ rejection but spare bone are beyond the scope of this article. At the present time, using the lowest possible dosages of glucocorticoid and calcineurin phosphatase inhibitors offers the best option. Currently, the most exciting areas of investigation involve agents that stimulate bone formation (growth hormone, growth hormone releasing peptide, PTH (1–34) or its analogues, the IGF family, including the IGF binding proteins, prostaglandins, particularly of the E series, and the TGF- β superfamily, including bone morphogenic protein). Such drugs are of theoretical value, particularly in the setting of glucocorticoid therapy where inhibition of bone formation is a major contributor to the bone loss. In this regard the recent study by Lane and colleagues in which subcutaneous PTH markedly increased lumbar spine bone loss in women with glucocorticoid-induced osteoporosis yielded encouraging results (180). Drugs that inhibit bone resorption, such as newer potent bisphosphonates, that can be given intravenously, may also prove effective. Prospective, controlled clinical trials are sorely needed, not only to evaluate existing regimens, but also to study these newer therapies.

SUMMARY AND CONCLUSIONS

Despite the inherent difficulties in interpreting clinical studies of skeletal effects of transplantation, there have been tremendous strides in elucidating the natural history and pathogenesis of transplantation osteoporosis. It is now clear that candidates for solid organ and bone marrow transplantation are at risk for bone loss and many already have osteoporosis or significant osteopenia and abnormal mineral metabolism. Prospective longitudinal studies have provided definitive evidence of rapid bone loss and fragility fractures, particularly during the first post-transplant year. The majority of transplanted patients sustain significant bone loss. Of even greater importance, fragility fractures occur in many organ transplant recipients, with incidence and prevalence rates ranging between 10% and 65%. Fractures often occur very early in the post-transplant course, sometimes within a few weeks of grafting. Moreover, vertebral fractures occur both in patients with low and those with normal pretransplant BMD, so that it is impossible to predict fracture risk in the individual patient. Early post-transplantation bone loss (<6 mo) is associated with biochemical evidence of uncoupled bone turnover, with increases in markers of resorption and decreases in markers of formation. Later in the post-transplantation course (>6 mo), concomitant with tapering of glucocorticoid doses, bone

formation recovers and the biochemical pattern is more typical of a high turnover form of osteoporosis.

During the past decade, there has been considerable progress in the diagnosis and treatment of many forms of osteoporosis. Nowhere is this more apparent than in the situation of glucocorticoid-induced osteoporosis; we now have several effective therapies for this debilitating condition, whereas prior to 1995, there were very few. There are drugs, available and approved by the FDA, that are capable of preventing bone loss in patients recently started on glucocorticoids and increasing bone mass in patients on long-term glucocorticoids. In addition, these therapies have been shown to reduce fracture risk by a sizeable percentage. Although few data are available from randomized, controlled clinical trials, both bisphosphonates and calcitriol show promise in the prevention of transplantation osteoporosis.

The National Osteoporosis Foundation, the American Association of Clinical Endocrinologists, and the American College of Rheumatology have all published practice guidelines that recommend BMD testing and appropriate intervention in all patients on long-term glucocorticoids and in those beginning glucocorticoids who are expected to remain on ≥ 7.5 mg for more than 1–3 mo. Moreover, the Bone Mass Measurement Act has provided for reimbursement of BMD testing in qualified patients who are taking or expected to be taking glucocorticoids. It has been recently stated that the expenses associated with monitoring bone density and metabolism, and treatment of osteoporosis and fractures in transplant patients represent an unwelcome burden in transplantation medicine (181). However, from a clinical rather than a financial perspective, it certainly seems humane to prevent unnecessary pain and suffering, particularly since it now is possible to accomplish this goal in many cases. It is highly likely that transplantation osteoporosis is, in large part, a preventable condition. In the coming decade, it will be important to firmly establish that this is the case by conducting randomized, controlled clinical trials.

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The Molecular Pharmacology of Osteoporosis

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INTRODUCTION

Physiology and Therapeutic Aims

Skeletal remodeling requires a balance between the resorption and formation of bone. Osteoclasts resorb bone, while osteoblasts are bone forming. These two processes are closely coupled but independently regulated by various hormones and cytokines. Osteoporosis is a disease characterized by excess osteoclastic activity compared to osteoblastic activity. Because of excess osteoclastic activity bones become fragile and may fracture, typically in the hip, spine, and wrist. Pharmacological therapy for osteoporosis should be aimed at preventing bone loss, whether the cause is involutional, postmenopausal, or secondary.

Osteoclasts are derived from hematopoietic progenitor cells. They are giant multinucleated (4–20 nuclei) cells found in bone alone. An excessive level of osteoclast activity causes inappropriate bone destruction in several bone and joint diseases including osteoporosis, Paget's bone disease, tumor-induced osteolysis, hyperparathyroidism, and rheumatoid arthritis. One therapeutic aim in these conditions is to prevent further bone loss (i.e., to decrease bone resorption). Either inhibiting osteoclast activity, decreasing their lifespan, or decreasing their formation can achieve this.

Osteoclast differentiation is controlled by a variety of cytokines including interleukin-1 (IL-1), IL-3, IL-6, IL-11, tumor necrosis factor (TNF), vitamin D₃, granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), and receptor activator of NF- κ B ligand (RANKL). RANKL with M-CSF can

induce osteoclast differentiation independent of stromal cells; however, stromal cells play a necessary role for osteoclast differentiation induced by other cytokines.

For osteoclasts to resorb bone, they must attach and create a tight seal of adhesion between their ventral surface and the bone matrix. Osteoclasts then must polarize with their ventral surface becoming a specialized organelle called the ruffled border with dense patches on either side called the sealing zone. The sealing zone is a region with contractile proteins and integrin receptors serving to attach the osteoclast to bone. Thus, the equivalent of an acidified lysosome-like cavity located between an osteoclast's ruffled border and the matrix is formed. Acidification, via a hydrogen pump (along with an Na^+/K^+ ATPase; $\text{HCO}_3^-/\text{Cl}^-$ exchanger; Na^+/H^+ exchanger), solubilizes the bone crystals to expose the matrix for dissolution. Digestive enzymes then dissolve the protein matrix and liberate previously deposited growth hormones and collagenases. Lysosomal enzymes secreted through the ruffled border include tartrate-resistant acid phosphatase (TRAP) and cathepsin K which degrades collagen and metalloproteinases including collagenase and gelatinase.

An encompassing model of resorption would include formation of the osteoclast, followed by polarization, creation of a ruffled border, induction of resorption, release of molecules from the matrix, and lastly apoptosis of the osteoclast. Therapeutic agents aiming to decrease bone resorption target mainly cancellous bone because it has the highest rate of bone matrix turnover. Current therapies with inhibition of bone resorption as their goal include estrogens and SERMs (selective estrogen receptor modulators), calcitonin, and bisphosphonates. These agents either block osteoclast proliferation, differentiation and fusion, or resorptive activity.

Osteoprogenitor cells are pluripotent bone stem cells capable of undergoing differentiation into osteoblasts upon stimulation. The transcription factor *Cbfa1* (Core-binding factor A1) and its possible mRNA splice variants are critical mediators of osteoblast formation (1). Osteoblasts are found in clusters along the surface of bone. They express steroid receptors for estrogen and vitamin D in their nucleus, as well as integrin and cytokine receptors. Endogenous stimulants of osteoblastic activity include fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), and transforming growth factor- β (TGF- β). Another therapeutic aim in osteoporosis and other bone diseases is to increase bone formation. Drugs intended to increase bone mass have the clinical advantage of increasing the fracture threshold. Agents with increased bone formation as their goal include fluoride and parathyroid hormone (PTH).

In the remodeling adult skeleton bone formation normally occurs only where bone resorption has previously occurred. The basis for this phenomenon is not completely clear but it probably reflects communication between the osteoblast and the osteoclast. For example, when osteoclasts resorb bone they release cytokines and growth factors such as TGF- β and IGF-1 that act on osteoblasts causing their activation and resulting deposition of new bone (2). While osteoclasts can engage in processes that activate osteoblasts, the reverse also holds true. Some stimulators of bone resorption such as parathyroid hormone-related protein, IL-1, and TNF act mainly on osteoblasts rather than osteoclasts (2). The osteoblasts then produce molecules that stimulate osteoclasts to resorb bone. Although these processes are coupled, they can occur independently of each other. For example, in some osteopetrotic mutants bone resorption is halted but bone formation continues unchecked, thus indicating that bone formation is not directly regulated by bone resorption (3).

Future targets for therapeutic action includes mechanisms that alter internal or external cell signaling or that directly introduce regulators of osteoclast and osteoblast function into bone. Cellular signaling targets will likely focus on the induction of osteoclast detachment and apoptosis, while those targeting osteoblasts and undifferentiated progenitor cells will likely focus on inducing proliferation and differentiation. Several interesting therapies are being explored for targeting proteins and drugs modulating osteoclast function to bone.

ESTABLISHED THERAPIES

Estrogen

Estrogen is the therapeutic mainstay for treating and preventing postmenopausal bone loss. For purposes of preventing bone loss, hormone replacement therapy should be considered as soon as possible after menopause for all women without contraindications; progestin should be administered concomitantly in women who have a uterus. Estrogens given immediately after menopause may cause some patients to gain new bone, as bone formation can transiently exceed resorption. If treatment is discontinued, rapid bone loss ensues.

Several findings are beginning to unravel the mystery of how estrogens interact with bone cells. One finding was the discovery of a second estrogen receptor isoform, the β isoform. While the α isoform predominates in the uterus, bone, and mammary glands, the β isoform is found in the cardiovascular, immune, and central nervous systems, as well as in the kidney, lung, urinary tract, and importantly bone (4). Estrogen receptors (ERs) are present in both osteoblasts and osteoclasts (5). ER β localizes with abundance in osteoblasts. Estrogen, through this receptor, may influence osteoblasts by altering the expression of bone morphogenic proteins (BMPs) and TGF- β .

Estrogens bind to nuclear estrogen receptors. Estrogen receptors have three interacting domains: the N-terminus domain is involved in hormone-independent activation function (AF1); the C-terminus ligand-binding domain (LBD) is important for ligand binding, dimerization and hormone-dependent activation (AF2); and the central DNA binding domain (DBD) (6,7). The two forms of the estrogen receptor (α and β) can dimerize as homodimers or as α , β heterodimers. Dimers, in turn, are capable of binding to estrogen response elements (EREs) present in DNA to stimulate transcription (8). This process occurs with the help of co-activators such as SRC-1, CBP, and RIP160. Additionally, estrogen can bind indirectly to AP-1 promoters via interactions with *fos* and *jun*. The action of estrogen receptors can be repressed with the corepressors SMRT and NcoR, among others (for a review of molecular estrogen action, see ref. 9). Synthetic antiestrogens acting through ER α may induce bone formation by regulating transcription of BMP-4, which activates genes enhancing osteoblast differentiation and therefore increases bone formation (10,11). Pharmacologically, synthetic estrogens preferentially activating only one ER subtype could be designed for the treatment of osteoporosis.

In osteoblasts estrogen acts to produce a spectrum of effects including progesterone receptor induction, enhanced procollagen production, and increased alkaline phosphatase expression (12–14). It also acts to induce mitogenesis and enhance insulin-like growth factor 1 (IGF-1) messenger ribonucleic acid (mRNA) expression (15). IGFs are autocrine enhancers of osteoblast function (16). Osteoblasts express IGF-1 and IGF-2, as well as 6 IGF binding proteins (IGFBPs), which regulate the actions of IGFs; IGFBP-5 has received attention for its ability to positively regulate osteoblast proliferation, although it also stimulates bone resorption via the formation of osteoclast (17). Additionally,

studies have shown that p120^{cas} is important in antiestrogen drug resistance (18); thus, it is inferable that p120^{cas} could have an intracellular signaling function preventing apoptosis of osteoblasts.

Estrogen may modulate the synthesis of resorption-inducing growth factors and cytokines by suppressing their transcription. For example, estrogen inhibits the production of IL-1, TNF- α , and IL-6 from stromal cells, monocytes, and lymphoid cells (19–21). This can be seen clinically in that post-menopausal women treated with estrogen have lower levels of IL-1, TNF- α , GM-CSF, and reduced bone loss (22). It is speculated that estrogen inhibits resorption by modulating NF- κ B signaling to prevent activation of the IL-6 promoter. This repression ability has recently been shown to be independent of the estrogen receptor's transcriptional induction capabilities (23). This has pharmacological ramifications in that even though these two processes (induction and repression of gene transcription) occur through the same domain (LBD), they can be regulated separately.

In osteoblasts, estrogen application stimulates rapid Ca²⁺ influx and phosphatidyl inositol biphosphate metabolism (24). These prompt actions likely occur through the activation of an unknown membrane receptor (not the ER) and the adenylate cyclase/cAMP pathway, which enhances Ca²⁺ influx across the L-type Ca²⁺ channel. Ca²⁺ mobilization has been found to occur from the endoplasmic reticulum, with the resulting formation of inositol 1, 4, 5-trisphosphate and diacylglycerol (25).

One possible explanation for estrogen's effects on osteoblasts is that estrogens acutely stimulate nitrogen oxide synthase (NOS) activity through the adenylate cyclase/cAMP/Ca²⁺ influx pathway. Estrogen-induced NOS activation has been shown in osteoblastic cells via the eNOS pathway (26). Nitric oxide (NO) production could explain the antiresorptive effects of estrogen, as NO causes osteoclasts to expediently contract and detach from their environment, as well as cease resorption. Against this theory is the fact that the observed effects of estrogen on NOS induction (minutes) are kinetically not sufficient to explain the rapid effects of estrogen seen with Ca²⁺ influx (seconds).

In osteoclasts, estrogen inhibits RANKL/M-CSF-induced AP-1 dependent transcription likely via direct regulation of *c-Jun* kinase (see Fig. 4B) (27). Recent in vitro work indicates that the expression of both *c-Jun* and *c-Fos* may be attenuated by estrogen by decreasing the activation of Jun N terminal kinase 1 (JNK1) (28). Through this mechanism and others, estrogen is able to inhibit the formation of functional osteoclasts. An estrogen deficiency causes increased osteoclast formation by enhanced phosphorylation of the nuclear protein Egr-1 in stromal cells. This results in increased stromal cell production of M-CSF, a cytokine crucial for osteoclast formation (see Fig. 1) (29). Estrogen application inhibits osteoclast formation via decreased levels of M-CSF. Another report has shown that in an estrogen deficient state the number of T-cells producing TNF- α is increased and that this (increased TNF- α) could explain the increase in resorption (30). Note in Fig. 2B that both RANKL and TNF- α signal to activate NF- κ B. Therefore, in addition to modulating molecules like M-CSF, osteocalcin, osteonectin, and osteopontin, estrogen can directly suppress RANKL-induced osteoclast differentiation. In summary, estrogen can inhibit osteoclast differentiation and resorption indirectly by regulating molecules such as M-CSF and NO, and directly by suppressing signaling from and/or transcription of RANKL, IL-1, IL-6, and TNF- α .

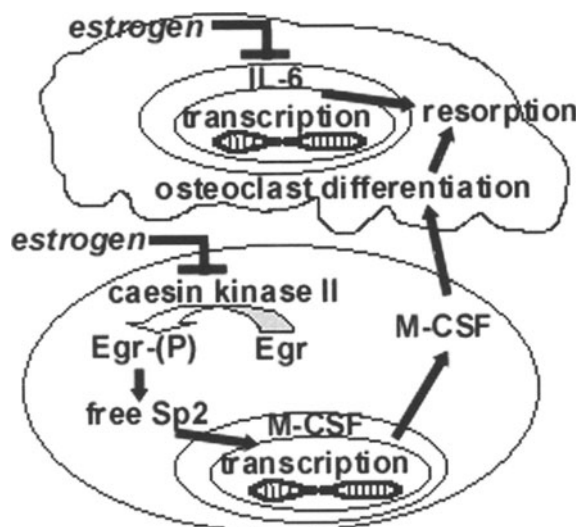


Fig. 1. Postmenopausal estrogen loss leads to casein kinase II activation in stromal cells, which in turn causes M-CSF transcription. M-CSF is a major inducer of osteoclast differentiation.

Bisphosphonates

Bisphosphonates (BPs) are stable analogs of pyrophosphate that inhibit bone resorption. They are used therapeutically in conditions characterized by increased bone remodeling namely Paget's disease of bone, hypercalcemia of malignancy, and osteoporosis (including glucocorticoid-induced osteoporosis) (31). The efficacy of bisphosphonates as preventive agents is not yet firmly established. However, they are FDA approved for the treatment of osteoporosis in postmenopausal women, and have been shown in these women to cause significant reductions of fracture incidence and gains in bone mineral density. Their main effects include decreased osteoclast progenitor development, decreased osteoclast recruitment, and induction of osteoclast apoptosis leading to inhibition of bone resorption (32–34). Because bisphosphonates are absorbed into newly synthesized bone, very high doses can inhibit bone mineralization. This effect is most notable in a Ca^{2+} -deficient state, thus clinically calcium supplements must be given with bisphosphonates to reduce the risk of mineralization defects.

In bisphosphonates the oxygen atom of the P-O-P (pyrophosphate) is replaced with a carbon atom resulting in a P-C-P bond. Their strong avidity to bone, similar to that of fluoride and tetracyclines, is related to their marked affinity for solid phase calcium phosphate. The two P-C bonds are resistant to enzymatic hydrolysis. Bisphosphonate variations are possible by changing the two lateral carbon side chains or by esterification of the phosphate groups. Of these two side chains, R_1 and R_2 , the R_1 side chain is usually a hydroxyl group to enhance the affinity of the compound for bone (35,36). Variations in the structure and conformation of the R_2 side chain determine the antiresorptive potency (35–37). Early bisphosphonates had a short R_2 side chain, such as " CH_3 " in etidronate or " Cl " in clodronate. These bisphosphonates are less potent than later bisphosphonates (e.g., pamidronate and alendronate), which have an amino-containing R_2 side chain (37). The most potent bisphosphonates (e.g., risedronate, zoledronate, and ibandronate) have a tertiary amino group as their R_2 side chain (37,38).

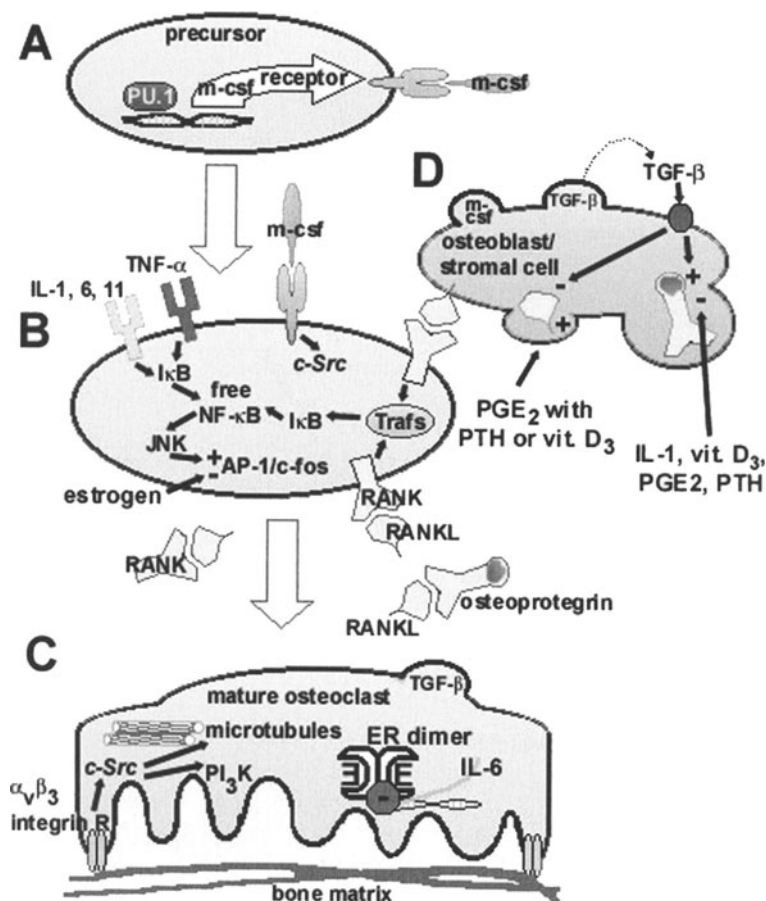


Fig. 2 (A) In osteoclast precursors, PU.1 encodes for the M-CSF receptor gene. Interaction of the receptor with M-CSF is essential for differentiation into the osteoclast lineage. (B) Interactions causing differentiation and enhanced survival of osteoclast precursors. Signaling from multiple sources converges on NF- κ B, which leads to alteration in gene transcription by AP-1/c-fos. (C) Interactions in mature osteoclasts showing a possible signaling pathway from the integrin receptor and negative regulation of IL-6 transcription by estrogen receptor dimmers. (D) The role of osteoblasts/stromal cells in controlling osteoclast differentiation. Note that osteoprotegerin is a negative regulator of osteoclast differentiation, while RANKL is a positive regulator.

Bisphosphonates have several possible modes of action. They are best known for their antiresorptive effects on osteoclasts. However, another premise important in certain types of osteoporosis is that bisphosphonates inhibit osteoblast and osteocyte apoptosis (39). In cell culture, this antiapoptotic action occurs through bisphosphonate-induced phosphorylation and activation of extracellular signal regulated kinases (ERKs), that in turn can enhance cell survival (39). In vivo, bisphosphonates are able to prevent glucocorticoid-induced osteoporosis by decreasing the prevalence of osteoblast and osteocyte apoptosis (39).

Regarding osteoclasts, bisphosphonates have been shown to induce apoptosis by inhibiting the mevalonate pathway and preventing posttranslational prenylation of GTP-binding proteins, including Ras (see Fig. 3) (40). Specifically, N-containing bisphosphonates (alendronate, risedronate, ibandronate, and pamidronate) have been found to inhibit the

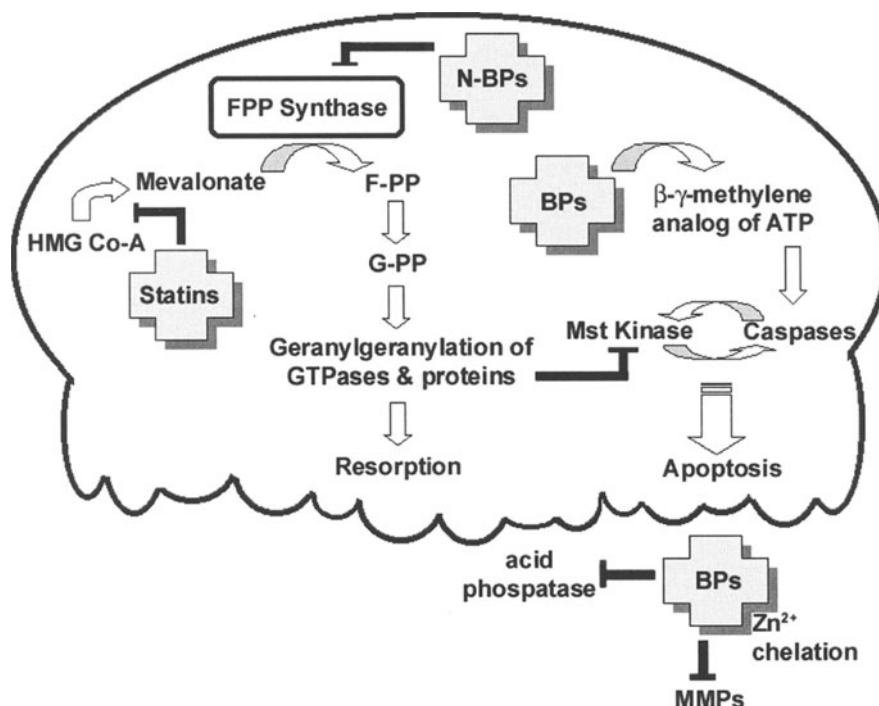


Fig. 3. The effects of bisphosphonates on osteoclasts and resorption. Statins inhibit the conversion of HMG Co-A to mevalonate. Nitrogen-containing bisphosphonates (N-BPs) inhibit the enzyme farnesyl diphosphate (FPP). Nonnitrogen-containing bisphosphonates (BPs) are metabolized to cytotoxic ATP analogs that induce apoptosis via the caspases. Additionally, bisphosphonates may chelate zinc and thereby inhibit matrix metalloproteinases (MMPs), and may also inhibit acid phosphatases.

isoprenoid biosynthesis pathway resulting in reduced geranylgeranyl diphosphate levels and subsequent inhibition of protein prenylation, as well as activation caspase-3 type proteases (37,41). Farnesyl diphosphate synthase has been identified as the mevalonate pathway enzyme inhibited by bisphosphonates (42). For alendronate, risedronate, and lovastatin, but not clodronate, induction of apoptosis occurs downstream of geranylgeranylation inhibition and is caused by caspase cleavage of Mst1 kinase (43).

The less potent nonaminobisphosphonates (e.g., clodronate, etidronate, and tiludronate) do not inhibit protein isoprenylation but rather are intracellularly metabolized to a cytotoxic, β - γ -methylene (AppCp-type) analog of ATP (see Fig. 3) (37,44). Their cytotoxic effects are through the intracellular accumulation of these AppCp-type metabolites.

Bisphosphonates may also interfere with matrix metalloproteinase (MMP) activity. Bisphosphonate-induced inhibition of MMP activity has been shown in bone metastasizing cancers to occur via phosphonate group chelation of zinc (45); a similar inhibition may occur for osteoclast proteinase activity (see Fig. 3). Another proposition is that bisphosphonates could inhibit the catalytic activity of the secreted acid phosphatase (46). In osteoclasts, we found that inhibition of acid phosphatase activity correlated with the antiresorptive activity of bisphosphonates. It is possible that bisphosphonates competi-

tively inhibit acid phosphatase action on bone and prevent the removal of pyrophosphate. Bisphosphonates have also been conjugated to estrogen to enhance specificity to bone.

Calcitonin

Calcitonin (CT) has been licensed for use in osteoporosis treatment for several years. High-turnover osteoporosis, such as occurs in the post-menopausal form may respond better to calcitonin than does the low-turnover form (47).

The utility of calcitonin as a therapeutic agent in states of accelerated bone resorption has also been documented in Paget's disease and hypercalcemia of malignancy. Trials in osteoporotic patients treated indicate that calcitonin causes a stabilization or modest short-term increase in bone mass, particularly in trabecular bone. Calcitonin may have the advantage of producing an analgesic effect and may be useful during postfracture periods.

The main action of calcitonin is on bone, although at pharmacological concentrations it may also increase renal calcium and phosphate excretion and 1,25-dihydroxycholecalciferol production (48). When infused into experimental animals with high bone turnover or into human subjects with Paget's bone disease, calcitonin causes a sharp fall in plasma Ca^{2+} . This action is caused by inhibition of osteoclast activity and hence a diminished Ca^{2+} efflux from bone to blood (49).

Calcitonin inhibits basal and stimulated resorption of intact bone in organ culture (50–55). In bone sections, calcitonin acts directly on the osteoclast to cause a rapid loss of the ruffled border (55–57). When applied for a longer term, there is a reduction in the number of osteoclasts in bone, an action that is relevant to its use in Paget's bone disease (49).

When applied in vitro to isolated osteoclasts, femtomolar concentrations of salmon calcitonin result in an acute cessation of cytoplasmic motility followed by gradual pseudopodial retraction (58). Calcitonin inhibits acid hydrolase secretion, Na^+ - K^+ -ATPase activity, and alters the localization of carbonic anhydrase and thus acid secretion in osteoclasts (see Fig. 4) (46,59,60). Additionally, calcitonin inhibits the release and synthesis of tartrate resistant acid phosphatase (TRAP) in osteoclasts (61).

To discern the post-receptor effect of calcitonin on the osteoclast, we developed a novel method to quantitate osteoclast motility. Several components of calcitonin action affect osteoclastic bone resorption independently (63). The more rapid quiescence (Q) component ($t_{1/2} \sim 15$ min) is characterized by cessation of motility, while the late retraction (R) component ($t_{1/2} \sim 27$ min) is characterized by pseudopodial retraction that may reduce resorption by decreasing the area of contact with the bone surface.

The Q and R effects of calcitonin are mediated by separate transduction pathways via distinct calcitonin receptor (CTR) subtypes (63). A cholera toxin-sensitive G_s protein mediates the cAMP-dependent Q component. The pertussis toxin-sensitive G_q protein mediates the R component via a rise in intracellular (Ca^{2+}). The CTR subtype that affects the Q component is activated by calcitonin and its related peptides amylin and calcitonin gene-related peptide (CGRP). In contrast, the subtype mediating the R effect is highly specific for calcitonin (64).

CTR subtypes have been cloned and sequenced. Several subtypes of the human calcitonin receptor hCTR have been discovered and result from alternative splicing of the primary mRNA transcript (65). The two most common human subtypes differ in the sequence by a 16 amino acid insert in the first putative intracellular domain. The hCTR-C1a, or insert-positive form, is the most prevalent and is capable of transducing intrac-

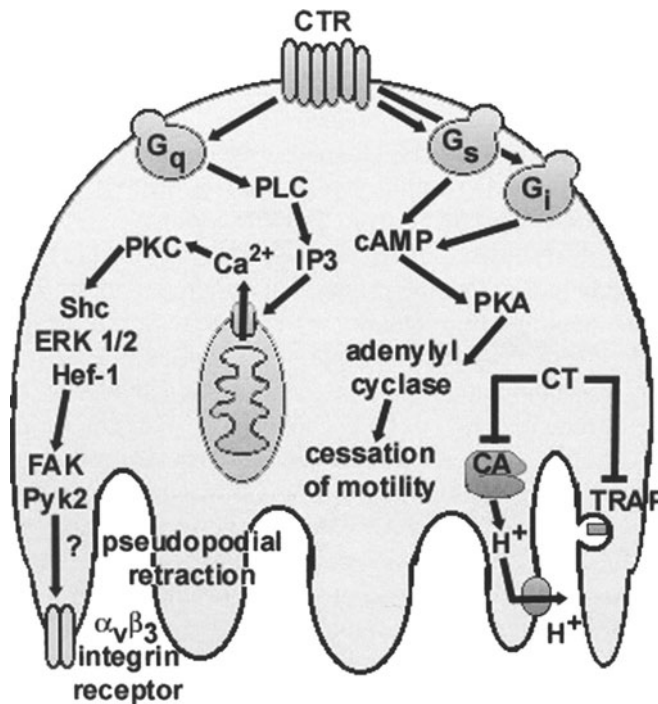


Fig. 4. Effects of calcitonin on osteoclasts. Signaling from the calcitonin receptor (CTR) activates the G_s/G_i proteins leading to cessation of motility, and activates G_q proteins leading to pseudopodial retraction. Calcitonin (CT) alters the location of carbonic anhydrase (CA) thus inhibiting acidification, and also inhibits the release of tartrate-resistant acid phosphatase (TRAP) (lower right corner).

cellular signals at least through G_s and G_q protein-coupled mechanisms (66,67). The calcitonin receptors are known to couple to G_s, G_q, and G_i, activating adenylyl cyclase, phospholipase C, and adenylyl cyclase, respectively. Inhibiting or depleting protein kinase C does not effect the activation of adenylyl cyclase in with the G_s-coupled response indicating that this signal likely occurs through the G_s/cAMP/protein kinase A pathway (68). This pathway, mediated through the third intracellular loop and C-terminal tail of the CTR (69), likely causes the observed cessation of motility. However, CTR signaling activating adenylyl cyclase and coupled to G_i is suppressed in the presence of activated protein kinase C (70). Thus, protein kinase C activation may act as a regulator of signaling for the G_i-coupled pathway. Signaling through G_q activates phospholipase C and PKC in osteoclasts. This activation, with resulting phosphorylation of *Shc* and ERK1/2 as well as Hef-1, likely induces pseudopodial retraction.

As part of the observed pseudopodial retraction, calcitonin likely regulates adhesion mechanisms in osteoclasts. Pyk2 is a major adhesion-dependent tyrosine kinase in osteoclasts that likely associates with *c-Src* via its SH2 domain (71). Engagement of the α_vβ₃ integrin, the major integrin present in osteoclasts, increases tyrosine phosphorylation of Pyk2 and leads to the formation of the sealing zone (71). The human enhancer of filamentation-1 (Hef-1 or CasL) is a focal adhesion-associated multiple-domain docking protein. It contains an SH3 domain that binds focal adhesion kinase (FAK) and Pyk2. Transduction from the calcitonin receptor that activates Hef-1 but not p130^{cas} is depen-

dent upon protein kinase C (and thus the G_q -coupled CTR), but independent of the G_s /cAMP/protein kinase A pathway (*see* Fig. 4) (72). Thus, activation of HEF-1 by calcitonin may cause interactions with Pyk2 and other molecules that regulate osteoclast adhesion, although this remains to be established.

Calcitonin also has effects in other tissues that relate indirectly to bone remodeling. The enzyme 25-hydroxyvitamin D3 1 α -hydroxylase (CYP27B1) catalyses the biosynthesis of 1 β , 25-dihydroxyvitamin D₃ from 25-hydroxyvitamin D₃ in renal proximal tubules. Calcitonin has been shown to increase the mRNA expression of the CYP27B1 enzyme and thus could help regulate the production of active vitamin D₃ (73).

With continuous therapeutic use of calcitonin, a decrease in bone responsiveness is observed. This is likely due to receptor down regulation in existing osteoclasts (74). New drug developments efforts could aim to increase receptor responsiveness to calcitonin. The seventh transmembrane domain has been shown to be essential for phospholipase C induction (i.e., G_q coupling) in the C1a isoform (75). Thus, drugs targeting one transmembrane domain may abolish certain functions while sparing others. Additionally, in a study on post-menopausal women with osteoporosis it was found that they had decreased calcitonin receptor mRNA levels compared to control subjects (76). It was noted that the prevalence of the hCTR-2 receptor isoform was inversely proportional to bone turnover rates (76). Because the different receptor isoforms may regulate different functions, drugs targeting one isoform over another may reduce side effects or preferentially affect forms of osteoporosis that have high or low bone turnover rates. This is highly speculative though.

POTENTIAL TARGETS FOR INHIBITION OF BONE RESORPTION

Interleukin-1 and Prostanoids

IL-1 has been shown to enhance growth and differentiation of osteoclast precursor cells and to stimulate mature osteoclasts to resorb bone (77). IL-1 is hypothesized to induce osteoclastogenesis by down regulating osteoprotegerin (OPG) (*see* Fig. 2D) (78). IL-1 also triggers NF- κ B production in certain cells. This may happen in the osteoclast via IRAK's (a kinase) and p62s (an intermediary protein) interaction with TRAF-6 (79). TRAF-6 is involved in IL-1 receptor signaling via its interaction with TAK1 and TAB1 (79,80). These actions remain to be established in the osteoclast, but could represent therapeutic targets for antagonist drug development.

Another action of IL-1 is stimulation of prostaglandin G/H synthase (PGHS) transcription, and thus the formation of prostanoids. IL-1 stimulates PGHS-2 expression in osteoblasts by CCAAT enhancer binding proteins (C/EBPs) β and δ -induced gene transcription (81). Prostanoids (prostaglandins E₂, PGE₂, and I₂, PGI₂) have been known to stimulate bone resorption both directly and indirectly. They may act directly (PGE₂ at least) on osteoclasts through prostaglandin receptors (EP₄ in particular) (82). Induction of resorption might happen by stimulation of PPAR δ / β in osteoclasts, which results in increased cathepsin K, carbonic anhydrase type II, and tartrate-resistant acid phosphatase gene expression (83). Inhibitors of PGE receptors seem promising for preventing bone resorption. An inhibitor of the EP1 subtype of PGE receptors was able to prevent osteoclast formation induced by PGE₂, PTH, IL-6, or 1,25-dihydroxy vitamin D₃ (84). Another mechanism by which prostaglandins may stimulate resorption is indirect, and involves stimulating interleukin-6 (IL-6) production from stromal cells and osteoblasts (85).

Additionally, prostaglandin (PGE₂) stimulation may be necessary for parathyroid hormone and vitamin D₃-induced RANKL expression on osteoblasts (*see* Fig. 2D) (86).

M-CSF

Macrophage colony-stimulating factor (M-CSF) plays an important role in osteoclast development, as its presence is required in most experimental models of osteoclastogenesis. When the M-CSF gene is mutated, as in the *op/op* mouse, osteoclasts fail to differentiate (87). M-CSF acts through the *c-fms* receptor on osteoclast precursors, and may act through this receptor to enhance the survival and chemotactic ability of mature osteoclasts (*see* Fig. 4A) (88,89). M-CSF application maintains NF- κ B and increases the mRNA expression of *bcl-2* and *bcl-X_L* (90). The likely reason that both M-CSF and RANKL are required for osteoclast survival is that M-CSF does not activate JNK kinase while signaling from RANK does, and conversely RANKL alone does not increase the mRNA expression of *bcl-2* and *bcl-X_L* while M-CSF does (90). Increased stromal cell production of M-CSF is caused by enhanced phosphorylation of the nuclear protein Egr-1 and is central to the mechanism by which estrogen deficiency increases resorption (*see* Fig. 1). Transcriptional regulation of M-CSF does not appear to be regulated by stimulation but rather by suppression. Deletion of the NF- κ B response element of the M-CSF gene increased basal promoter activity, thus implying that NF- κ B may act as a negative regulator of M-CSF production in stromal cells and osteoblasts (91).

TNF- α

TNF- α stimulates proliferation and differentiation of osteoclast precursors in the marrow and potentiates the osteoclast-inducing effects of IL-1. TNF- α effects are likely involved in hypercalcemic states (92). One possible mechanism for increased resorption is that TNF- α via IL-1 triggers enhanced prostaglandin synthesis. Osteoclast progenitor cells express the TNF receptors p55 and p75. The proliferation and differentiation effects of TNF- α are inhibited completely by an anti-p55 antibody and partially by an anti-p75 antibody (93). This may be because the p55 receptor activates NF- κ B, where the p75 receptor does not (94). (See section on NF- κ B for its effects) Thus, drugs targeted at inhibiting the p55 TNF- α receptor may be able to quell osteoclastic bone resorption. However to stop resorption by differentiated osteoclasts via inhibiting their pit formation, IL-1 may also have to be modulated (95). TNF- α is important in the activation of NF- κ B and molecules controlling this interaction may include RIP (a death domain kinase) and p62 (an adapter protein) (79).

Interleukin-6

IL-6 (produced by stromal cells, macrophages, osteoclasts, and osteoblasts) induces osteoclast precursors to differentiate by binding to their IL-6 receptors and signaling through gp130 (96,97). IL-6 stimulation likely serves as a costimulator for other hormones and cytokines such as vitamin D₃ and may prevent apoptosis of osteoclasts (33). IL-6 expression in osteoblasts and stromal cells is increased by several stimuli including parathyroid hormone (PTH), IL-1 TNF- α , vitamin D₃, and PGE₂ (96,98 99). PTH and TNF- α stimulate IL-6 production via a PKC-dependent pathway, while IL-1 functions through a different pathway (for a further explanation see section on PTH). We have shown that a high extracellular (Ca²⁺) transduced via a Ca²⁺-sensing receptor inhibits

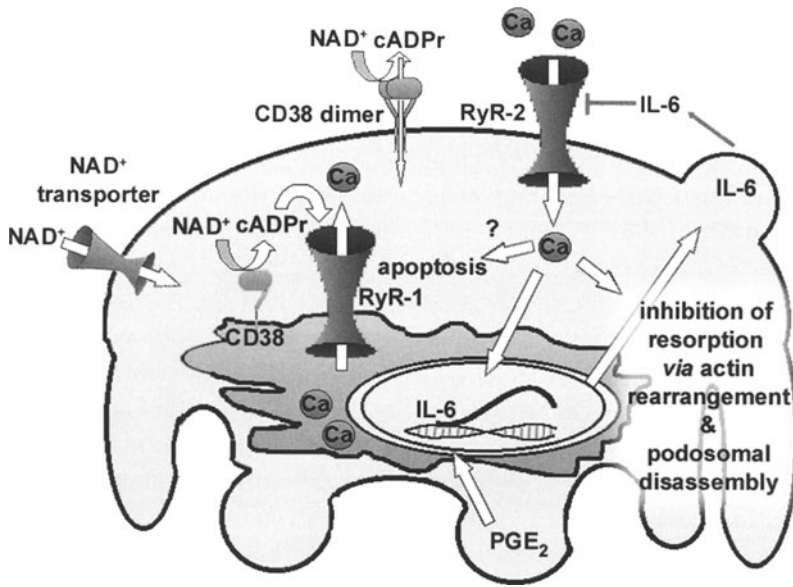


Fig. 5. Possible mechanisms causing increased intracellular (Ca^{2+}). RyR-2 present on the plasma membrane may serve as a Ca^{2+} influx channel. NAD^+ may serve to induce Ca^{2+} release by first being converted to cADPr that then activates RyR-1 Ca^{2+} channels present on the ER membrane. Ca^{2+} influx initially halts resorption by causing actin rearrangement and podosomal disassembly. However, Ca^{2+} influx also stimulates IL-6 transcription; IL-6 is capable of inhibiting Ca^{2+} -sensing and may allow resorption to resume.

osteoclastic resorption and causes increased IL-6 and IL-6 receptor transcription (see Fig. 5) (100). We found that IL-6 then attenuated Ca^{2+} -sensing and allowed resorption to continue in spite of a high extracellular (Ca^{2+}). Postmenopausal estrogen loss causes an increase in PTH-induced IL-6 production and thus bone resorption (101,102). Additionally, IL-6 may contribute to postmenopausal bone loss through osteoclast recruitment (103). Pharmacologically, targeting IL-6 receptor signaling may be difficult because both IL-6 receptors and gp130 are present in several cell types. Recombinant forms of gp130 that block IL-6 signaling have shown marginal progress for other disease states but ideas similar to this have yet to be applied to bone diseases.

RANK/RANKL

The receptor activator of NF- κ B and the receptor activator of NF- κ B ligand, called RANK and RANKL, respectively, are critical to osteoclast development and the stimulation of resorption. RANKL (also called TRANCE, ODF, and OPGL) is a ligand, produced by stromal cells and osteoblasts, that interacts with its receptor on osteoclasts, called RANK. This interaction is essential for stimulating osteoclastogenesis and bone resorption (104). RANKL is present on the membrane of osteoblasts, stromal cells, and T-cells, as well as secreted as a soluble molecule in the bone microenvironment by these cells (see Fig. 2) (105). It has been shown that RANKL induces differentiation of osteoclast precursors away from a macrophage lineage to that of an osteoclast lineage (106). Several molecules (such as interleukin-11, IL-11, and prostaglandins) can induce expression of RANKL on osteoblasts and stromal cells and subsequently cause osteo-

clast differentiation (107). RANKL-induced NF- κ B activation is inhibited by activation of the osteoclast peroxisome proliferator-activated receptor- γ (PPAR- γ) (108); agonist activation of the PPARs may represent a way to suppress osteoclast differentiation induced from RANKL.

In osteoclasts, RANK interacts with TNF receptor-associated factors (TRAFs) via its intracellular domains. After recruitment to RANK, TRAFs initiate complex formation that causes translocation of NF- κ B from the cytoplasm to the nucleus and activation of *c-Jun* N-terminal kinase (JNK) among others (see Fig. 2B). Thus, RANK via TRAFs controls the activation of NF- κ B (109). RANK contains a binding site for TRAF-6, which subsequently induces the activation of NF- κ B (110). MAP kinase kinase (MKK) 6 and p38a MAP kinase also likely play a role in transducing this signal for osteoclast differentiation (111).

NF- κ B

Nuclear factor- κ B, known as NF- κ B, is a key osteoclast differentiation signal. NF- κ B exists in the cytoplasm as either homo- or heterodimers bound to each other via a Rel-homology domain (RHD). The NF- κ B dimer can be composed of any two of five various protein subunits, although the classical NF- κ B dimer is composed of RelA and NF- κ B1 (112). The I κ Bs are proteins that bind to the dimer and prevent translocation into the nucleus from the cytoplasm. Signaling pathways such as IL-1, TNF- α , and RANK lead to the phosphorylation of I κ Bs and their subsequent ubiquitin-related degradation (112). When NF- κ B1 and NF- κ B2 are both knocked out, a defect in osteoclast differentiation occurs resulting in osteopetrosis (113). Potential therapeutic uses of this pathway remain to be established as the NF- κ B subunits and downstream activators of NF- κ B, such as the I κ B kinases (IKKs), have essential functions in multiple cell types.

c-Src

c-Src is a membrane tyrosine kinase that plays a role in inducing osteoclasts to resorb bone. *c-Src* knockout mice have osteoclasts that are dysfunctional (114). *c-Src* may act to allow cortactin-regulated adherence of osteoclasts to bone. In the *c-Src* pathway, activation of Ral-GTPases have been coupled to subsequent transcription and cytoskeletal rearrangements through tyrosine phosphorylation of Stat3 and cortactin, respectively (115). This same group also showed that *c-Src* kinase activity is used differently by individual extracellular stimuli thus suggesting that different *c-Src* isoforms could be involved in various signaling cascades; this fact may prove useful in designing a *c-Src* inhibitor that shows preferential treatment for a particular pathway. Bone matrix recognition results in *c-Src* association with microtubules (see Fig. 2C). This likely happens *via* the integrin α v β 3. Signaling from this integrin involves *c-Src*, as well as phosphatidylinositol 3-kinase (PI₃-K), Pyk2, and p130^{cas} among others (71). To this end, *c-Src* knockout mice show abnormalities in their localization of α v β 3 integrins and thus spreading, attachment, and formation of a sealing zone (116).

Additionally, *c-Src* may be important for the chemotactic ability of osteoclasts. M-CSF stimulates osteoclast motility *via c-Src*. Recently M-CSF stimulation at the *c-fms* receptor was found to cause the activation of *c-Src*; activated *c-Src* subsequently interacts *via* its SH3 domain with the p⁸⁵ regulatory domain of PI3-K causing translocation of PI3-K to the cell periphery (117). Therefore, *c-Src* may play a role in osteoclast adherence to the bone matrix *via* M-CSF stimulated chemotaxis and α v β 3 integrin stimulated adherence.

Inhibitors of *c-Src* have been developed. Substituted 5,7-diphenyl-pyrrolo[2,3d]pyrimidines potently inhibit *c-Src*'s tyrosine kinase function (118). However, it remains to be seen whether these compounds will be effective in reversing or halting osteoporosis. Another approach to decreasing $\alpha_v\beta_3$ integrin stimulated adherence is blocking vascular endothelial growth factor (VEGF). Evidence exists that signaling from VEGF receptors activates integrin mediated adhesion in epithelial cells (119), and supports osteoclast differentiation and bone remodeling (120). An in vivo therapy being explored is neutralization of VEGF with monoclonal antibodies; one group has shown inhibition of bone resorption in mice using this technique (121).

Interleukins 4 and 18

Interleukin 4 (IL-4) is produced by activated T-cells and inhibits the formation of osteoclasts (122). Therapeutic implications of IL-4 show promise. Local overexpression of IL-4 in mice, introduced by a recombinant human type 5 adenovirus vector, inhibited osteoclasts likely through decreased levels of RANKL, cathepsin K, and IL-6 (123).

Interleukin-18 (IL-18) inhibits osteoclast formation indirectly. IL-18 induces GM-CSF production by T-cells, which then inhibits osteoclasts (124). IL-18 is likely involved in inflammatory processes as its production may be induced by histamine and modulated by corticosteroids.

NEW STRATEGIES FOR STIMULATION OF BONE FORMATION

Parathyroid Hormone

A high continuous dose of parathyroid hormone (PTH) or the similarly effective parathyroid hormone related protein (PTHrP) have been known to cause increased bone resorption. However, PTH has recently been spotlighted because its application at low, intermittent doses has the anabolic effect of increasing bone density. For example, small doses of PTH (1–34) have been shown in studies to cause an increase in trabecular bone volume and spinal bone mass. The major drawback with anabolic PTH therapy is its parenteral administration and cyclic dosing.

Molecularly, PTH acts on osteoblasts to increase levels of TGF- β 1 and TGF- β 2 via the activation of PKC or PKA pathways, respectively (*see* Fig. 6) (125). The effects of PTH signaling pathways in osteoblasts are not clear-cut. It has been shown that in osteoblasts PTH-induced activation of both the PKC and PKA pathways leads to IL-6 production, and thus bone resorption and osteoclast differentiation (126). This may explain the observed effect of stimulation of osteoclast fusion and formation of mature multinucleated osteoclast and thus bone resorption upon PTH application. PTH may increase matrix metalloproteinase (MMP-2 and MMP-9) activities (127).

PTH has been shown to regulate the expression of osteonectin, osteopontin, and osteoprotegerin (128–130). These proteins may account for some of PTH's observed anabolic effects on bone. A possible mechanism is that PTH stimulates TGF- β production, which consequently results in changes in osteonectin and osteopontin mRNA stability as well as osteoprotegerin production. More evidence needs to be gathered for the effect of in vivo PTH administration and the regulation of these proteins.

One way of targeting induction of PTH's anabolic effects on bone has been the development of antagonists (calcilytics) for the parathyroid Ca^{2+} sensing receptor. Antagonizing the Ca^{2+} sensing receptor imitates a state of hypocalcaemia and stimulates

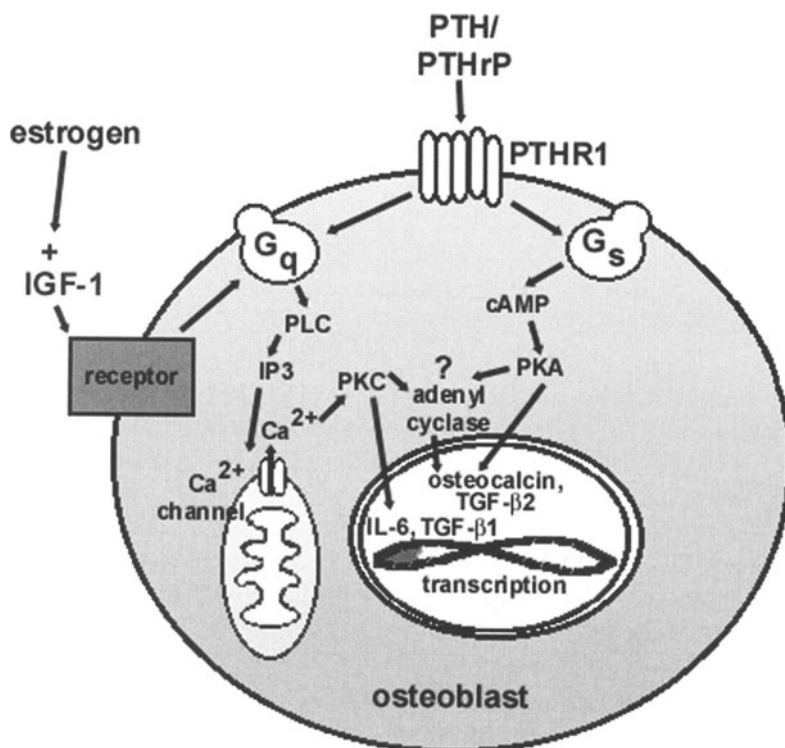


Fig. 6. Intracellular signaling from the parathyroid hormone receptor-1 (PTHR1). PTHR1, stimulated by PTH and PTHrP, can activate a G_q-coupled pathway that leads to increases in (Ca²⁺) with resulting activation of PKC and gene transcription. Alternatively, PTHR1 can activate a G_s-coupled pathway that leads to activation of PKA and transcription of the same or different genes.

PTH secretion. Ideally, the calcilytic should be a short acting orally administered substance that induces only a transient rise in PTH. A study with such a compound found that whereas bone turnover increased, bone mineral density did not (131). However, in the same study it was noted that bone resorption was reduced with coadministration of a calcilytic with estrogen, resulting in increased bone mass. Estrogen has a range of effects that increase bone formation including causing increased transcription of IGF-1 in osteoblasts (132).

Another way of targeting the induction of PTHs anabolic effects on bone may be by using Ca²⁺ sensing receptor agonists (calcimimetics) to intermittently decrease PTH levels. One study found an increase in bone mineral density and cancellous bone volume using such a compound (133). Thus, it appears that transient fluctuations, either increasing or decreasing PTH levels over time lead to increased bone volume and/or turnover.

Transforming Growth Factor-β

Transforming growth factor-β (TGF-β) is secreted by osteoblasts and osteoclasts and acts as an autocrine-stimulating factor for osteoblasts. It causes bone formation through enhanced chemotaxis, proliferation, and differentiation of osteoblasts. TGF-β-induced increases in osteoblast proliferation are independent of the differentiation state (134). TGF-β has three isoforms (β1, β2, and β3). They are mostly concentrated in bone with

the major isoform expressed by osteoblasts being $\beta 1$ and that by osteoclasts being $\beta 2$. Because of their localization and stimulatory effects on osteoblast proliferation, TGF- β analogs and TGF- β receptor (I and II) agonists represent potential pharmaceutical targets for increasing bone formation.

TGF- $\beta 1$ inhibits the proliferation and fusion of osteoclast precursors to form multinucleated cells and likely induces apoptosis of osteoclasts indirectly by up regulating osteoprotegerin (*see* Fig. 2D) (78). However, some reports have been contradictory (135). Likewise, TGF- $\beta 1$ down regulates expression of RANKL from stromal cells and osteoblasts, further inhibiting osteoclast differentiation (128). The stability of collagen and fibronectin mRNAs is increased in the presence of TGF- β , suggesting that it is directly capable of enhancing bone formation (129). TGF- β can also regulate osteonectin (also known as secreted protein rich in cysteine [SPARC]) expression; osteonectin is an extracellular matrix glycoprotein that can stimulate angiogenesis, the production of matrix metalloproteinases, and cell-matrix interactions by binding to collagen and hydroxyapatite (129,136). Thus, TGF- $\beta 1$ accomplishes two actions, osteoblast induction and osteoclast inhibition with the end result of increased bone formation. Pharmacologically, TGF- $\beta 1$ given to promote bone healing in rabbit skull caps lead to an increase in bone density (137). However, clinical trials of TGF- β may have the stumbling block of declining renal function with sustained administration (138).

Vitamin D₃

Calcitriol or 1,25-(OH)₂D₃ is the active metabolite of vitamin D₃. The primary regulators of calcitriol production are parathyroid hormone, calcitriol itself, and dietary intake of calcium and phosphate (139). 1,25-(OH)₂D₃ has the following major effects: it increases Ca²⁺ absorption from the intestine, it reduces parathyroid hormone (PTH) levels, and it decreases PTH-mediated bone resorption. Exposing osteoblasts to TGF- β results in increased expression of 1,25-(OH)₂D₃ receptors, which are nuclear receptors that act via binding to distinct vitamin D response elements (VDREs). Vitamin D receptors (VDRs) in the presence of TGF- β cause the expression of type I collagen and alkaline phosphatase (AP) by osteoblasts (134). Without TGF- β the case may be reversed, as it was observed that 1,25-(OH)₂D₃ inhibited type I collagen synthesis in rats by reducing steady state levels collagen mRNA (140).

In osteoblast precursors, VDR signaling inhibits *Cbfa1* expression; since *Cbfa1* is a transcription factor controlling osteoblast differentiation, application of 1,25-(OH)₂D₃ may inhibit osteoblast differentiation (141). Thus, any anabolic effects of 1,25-(OH)₂D₃ likely result from increases in osteoblast function. However, the study of 1,25-(OH)₂D₃ is complicated by species differences. While application of 1,25-(OH)₂D₃ upregulates the expression of the osteoblast-specific protein osteocalcin in rats and humans, in mice it inhibits osteocalcin expression (142).

1,25-(OH)₂D₃ may regulate osteoblastic secretion of C-type natriuretic peptide (CNP), which regulates expression of alkaline phosphatase and osteocalcin and induces the formation of mineralized nodules. Because 1,25-(OH)₂D₃ treatment significantly increases natriuretic peptide receptor-C (NPR-C) expression and NPR-C expression determines the biological availabilities of NPs, 1,25-(OH)₂D₃ could regulate the expression of alkaline phosphatase and osteocalcin through NPR-C (143).

L-type voltage-sensitive calcium channels (L-VSCCs) have been identified in osteoblasts and represent a further way in which 1,25-(OH)₂D₃ may regulate osteoblast func-

tion. The L-type VSCC (particularly essential is the α_{1c} subtype) may serve as the molecular transducer of a $1,25-(OH)_2D_3$ action that results in Ca^{2+} influx (144) but the physiological significance of this non-genomic action is unclear.

Binding of $1,25-(OH)_2D_3$ results in a conformational change in VDR that enhances the interaction of VDR with retinoid X receptor (RXR). VDREs in genes are recognized by an active VDR-RXR heterodimer. AF-2 domains in both VDR and RXR facilitate interactions with coactivators such as steroid receptor coactivators-1 (Src-1) and glucocorticoid receptor interacting protein (GRIP1). A new 170kD coactivator specific for osteoblasts has been identified (145) and could represent an osteoblast specific target for drug development.

Other vitamins may also be important in bone remodeling. There is some evidence that vitamin K_2 when given in vivo reduces the formation of osteoclasts, possibly by inhibiting the effects of M-CSF (146). Vitamin K_2 may also promote $1,25-(OH)_2D_3$ -induced mineralization (147) and enhance osteocalcin accumulation in the extracellular matrix of human osteoblasts (148). It has been proposed that a vitamin K_2 nuclear binding protein/receptor with a molecular structure close to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) exists in the osteoblast (149). This binding protein could represent a target for activating vitamin K_1 and K_2 analogs that enhance osteoblast formation.

Nitric Oxide

Nitric oxide (NO) causes osteoclasts to rapidly contract, detach from their environment, and cease resorption. Superoxide (O_2^-), which metabolizes NO, is overproduced by osteoclasts during bone resorption and may reduce the effects of NO. NO production occurs in response to a number of stimuli including interferon- γ (IFN- γ) with TNF- α , and fluid shear stress, among others (150). In osteoclasts, NO activates guanylate cyclase and cGMP-dependent protein kinase (G-kinase); G-kinase through a cascade reduces membrane HCl transport (151). Thus, there is evidence that NO is a negative regulator of osteoclast activity. However, evidence also suggests that NO derived from the iNOS pathway may play an important role in IL-1 signal transduction (152). Thus, NO could also positively regulate bone resorption.

Osteoprotegerin

Osteoprotegerin (OPG) is a member of the TNF receptor family produced by osteoblasts. OPG is a soluble, secreted decoy receptor that binds to the receptor activator of NF- κ B ligand, RANKL (153) (see Figs. 4B,D). RANKL, as discussed above, is a ligand produced on stromal cells and osteoblasts that interacts with its receptor on osteoclasts and osteoclast precursors, RANK (receptor activator of NF- κ B). This interaction stimulates osteoclastogenesis and bone resorption. OPG binds RANKL, thus inhibiting osteoclastogenesis and bone resorption. Stimulators of osteoclast formation, such as $1,25-(OH)_2D_3$, PGE_2 , parathyroid hormone, and IL-1, all decrease stromal cell and osteoblast production of OPG (78) (see Fig. 2D). Both RANKL and OPG production by osteoblasts is increased by the transcription factor *Cbfa1* (154,155). *Cbfa1* is modified depending on other extracellular signals (such as PTH-induced PKA-dependent transactivation of *Cbfa1*) and therefore could directly regulate osteoblast specific genes and hence osteoblast differentiation, as well as regulate osteoclast formation by modulating the ratio of RANKL to OPG (156,157). A similar therapy may be developed for osteoporotic patients.

Ca²⁺ Receptors

Extracellular Ca²⁺ can be sensed by osteoclasts and osteoblasts through Ca²⁺-sensing receptors. This likely occurs through either a traditional G-protein coupled Ca²⁺ receptor, or, as we have shown, through a ryanodine receptor (RyR) (158–160). High extracellular (Ca²⁺) inhibits osteoclastic bone resorption. Ca²⁺-induced inhibition of resorption occurs in osteoclasts by actin reorganization and podosomal disassembly (see Fig. 5) (158,161). Actin reorganization likely occurs through the tyrosine kinases *c-Src* and FAK, as inhibitors of tyrosine kinases thwart actin reorganization and osteoclast resorption activity (162). Inhibition of the tyrosine kinases leads to Ca²⁺ influx, which is insensitive to dihydropyridine (DHP) but sensitive to La³⁺ and Ni²⁺ application. Because extracellular application of cations attenuates Ca²⁺ influx occurring through plasma membrane RyRs (163), it is conceivable that this process is occurring through plasma membrane RyR Ca²⁺ influx channels.

High extracellular (Ca²⁺) also induces osteoclast apoptosis (164); Ca²⁺ influx controls this Ca²⁺-induced osteoclast apoptosis. RyRs and IP₃Rs are found on the nuclear membrane as well as on the plasma membrane (165). It remains to be determined what RyR receptors are activated in the induction of apoptosis. Three RyR isoforms exist; isoforms 1, 2, 3 are expressed in skeletal, cardiac, and smooth muscle or brain, respectively. The dominant form expressed in the osteoclast's nucleus and endoplasmic reticulum is RyR-1, and on the osteoclast's plasma membrane is RyR-2 (166). RyRs likely will have multiple roles in bone resorption depending upon other stimuli. For example, in osteoclasts Ca²⁺ inflow through RyRs is important for stimulating IL-6 production, and that RyRs gate Ca²⁺ release in response to NAD⁺ application (see Fig. 5) (167). In osteoblasts, cytosolic and nuclear Ca²⁺ influx, possibly through RyRs or IP₃Rs, could be an important component of integrin stimulated adhesion mechanisms (165,168).

EXPERIMENTAL STRATEGIES

Bone-Specific Targeting

Strategies targeting drugs specifically to bone would be beneficial in reducing systemic side effects. To this extent, one approach has been using drugs targeted to hydroxyapatite (HA). HA has the advantage of existing only in hard tissues such as bones and teeth. However, because teeth are stable after their formation, only bone will provide for the release of drugs adsorbed to HA. Bisphosphonate-conjugated estrogens have been used to limit the distribution of estrogen preferentially to bone (169). These conjugated compounds could potentially improve compliance by minimizing adverse effects and reducing medication frequency.

Taking this idea one step further, amino acid sequences that cause preferential targeting to HA, such as a repeating Asp or Glu sequence, has been combined with various drugs. It was shown that fluorescein isothiocyanate (FITC), when conjugated to a repeating sequence of six Asp's and given subcutaneously, was excreted from most tissues within 24 h. However, in bone it had a half-life of 14 d (170).

Another small peptide sequence that could be used to target drugs to bone is the HIV-TAT sequence. HIV-TAT facilitates entry of proteins into cells. Conjugating the HIV-TAT sequence to Rho proteins allowed easy transduction transfection into osteoclasts and induced podosome formation (171,172). Although one could easily imagine conjugating bone building or antiresorptive drugs to HIV-Tat, it must be considered that when

injected intraperitoneally, these conjugates will enter all types of cells. Future directions might consider conjugating both the HIV-Tat sequence and the Asp/Glu repeating sequence to enhance both entry and specificity of protein-based drugs.

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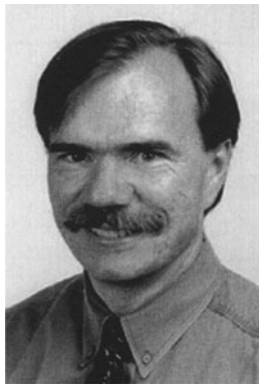
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